THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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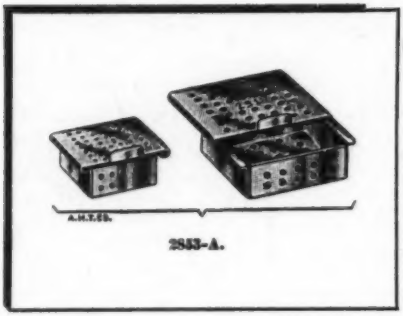

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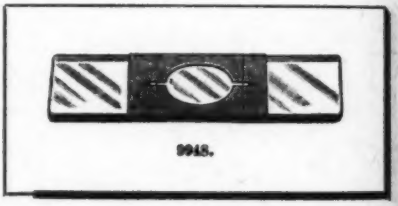

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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:00 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 9:00 A.M. Special

meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE TREASURER

The following tables reflect the financial position of the Laboratory.

The first table summarizes the position of the securities in the Endowment Funds. The market value of January 1, 1954, was $1,153,995.88 in comparison to the market value shown on January 1, 1953, of $1,166,063.74. The average yield on the securities was 4.77% on book values and 4% on market values, or slightly higher than was the case in 1952.

The second table gives the Balance Sheet of the Laboratory as of December 31, 1953. It reflects the entries which were necessary in connection with the taking-by-condemnation proceedings by the U. S. Navy of the Bar Neck property. While the final settlement of this matter is still subject to negotiation or litigation, $75,000 was deposited in the courts and paid over to the Laboratory. This resulted in our reducing the land and buildings by the amount at which this property had been carried and "other investment securities" were increased by about $75,000 due to the investment of these funds. As the matter has not been finally settled an item is carried on the liability side of $55,247.08 pending final determination; this item representing the difference between the original book value of the property and the amount paid to the Laboratory by the Government, and will be further adjusted later to reflect the actual total amount finally received from the Government for the property and to cover expenses incurred by the Laboratory in the condemnation proceedings.

The third table is a condensation of the auditor's income and expense statements for the past five years. As I noted last year, this table points out strongly the need of the Laboratory for additional income and the importance of continued efforts by everyone connected with the Laboratory to secure special donations each year. Your officers and the Executive Committee are giving this matter particular attention and will undoubtedly report to you from time to time. It is obvious that without these special donations, it would have been impossible for the Laboratory to continue to make available the facilities and services necessary for biological research and instructions.

The other tables submitted call for no comment.

Endowment Funds

January 1, 1954

	Book Value	%	Market Value	%
Bonds				
U. S. Government	$268,772.56	27.75	$ 260,944.00	22.62
Railroad	54,613.02	5.63	57,560.00	4.98
Public Utility	90,488.75	9.34	86,350.00	7.48
Industrial	139,128.13	14.37	135,100.00	11.71
Total Bonds	553,002.46	57.09	539,954.00	46.79
Preferred Stocks	103,805.13	10.72	96,816.00	8.39
Common Stocks	310,953.08	32.11	516,390.00	44.75
Total Securities	967,760.67		1,153,160.00	
Principal Cash	835.88	.08	835.88	.07
Totals	$968.596.55	100.	$1,153,995.88	100.

The average yield on the securities was 4.77% on book value, 4.00% on market value.

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1953

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of the Hanover Bank, New York, Trustees		$ 968,596.55	
Securities and Cash in Minor Funds		21,701.36	$ 990,297.91
Plant Assets:			
Land		$ 97,030.04	
Buildings		1,490,726.83	
Equipment		338,588.94	
Library		446,071.11	
		$2,372,416.92	
Less Reserve for Depreciation		885,781.84	$1,486,635.08
Current Assets:			
Cash		$ 23,352.94	
Mortgage Note Receivable		2,200.00	
Accounts Receivable		42,341.67	
Inventories:			
Supply Department	$ 52,565.88		
Biological Bulletin	17,014.61	69,580.49	
Investments:			
Devil's Lane Property	37,510.74		
Stock in Gen. Biol. Supply House, Inc.	12,700.00		
Other Investment Securities	122,149.39		
Retirement Fund	24,017.72	196,377.85	
Prepaid Insurance		6,808.33	
Items in Suspense (Debits)		3,160.86	343,822.14
			$2,820,755.13

Liabilities

Endowment Funds:			
Endowment Funds	$ 965,729.75		
Reserve for Amortization	2,866.80	$ 968,596.55	
		21,701.36	$ 990,297.91
Plant Funds:			
Mortgage Note Payable		5,000.00	
Donations and Gifts	1,409,441.81		
Other Investments in Plant from Gifts and Current Funds	72,193.27	1,481,635.08	1,486,635.08
Current Liabilities and Surplus:			
Accounts Payable		36,593.15	
Items in Suspense (Credits)		2,939.37	
Bar Neck Settlement Account		55,247.08	
Current Surplus		249,042.54	343,822.14
			$2,820,755.13

MARINE BIOLOGICAL LABORATORY

Five Year Condensed Income and Expense Statement

Reference—Exhibit B and B–1 Auditors' Reports—1949 through 1953

	1953	1952	1951	1950	1949	Total
Income from Endowments	$ 46,261.43	$ 44,115.00	$ 44,898.43	$ 46,431.59	$ 40,347.15	$ 222,053.85
Income from Investments, Including Retirement Fund and Mortgage Rec.	23,195.75	21,635.50	23,184.86	22,844.19	22,593.55	113,453.85
Income from Fees and Dues, including Instruction, Research, Library	56,852.85	46,695.76	46,633.00	47,888.61	44,921.33	242,991.55
Income from Mess, Dormitories, Supply, Biol. Bulletin, Sundry—Net	28,289.49	19,690.73	12,943.63	21,601.35	27,499.18	110,024.38
Income from Rents, including Bar Neck	.....	7,333.26	8,047.42	7,090.89	6,330.00	28,801.57
TOTAL ORDINARY INCOME....A	154,599.52	139,470.25	135,707.34	145,856.63	141,691.21	717,324.95
Income by Special Donations	49,049.00	65,242.69	77,730.00	43,130.66	37,459.45	272,611.80
TOTAL INCOME....X	$203,648.52	$204,712.94	$213,437.34	$188,987.29	$179,150.66	$ 989,936.75
Buildings, Grounds, and Other Property Expense	$ 55,159.57	$ 51,020.70	$ 56,353.84	$ 53,066.36	$ 41,591.16	$ 257,191.63
Research Service Expense—Apparatus and Chemical Rooms	33,613.24	24,312.98	25,492.99	25,244.54	23,901.70	132,565.45
Instruction, Research and Library Expense	45,968.79	34,632.76	34,083.50	34,077.76	30,587.06	179,349.87
General Expenses, Administration, Pension Fund, Bank Charges, etc.	42,790.76	39,396.93	41,375.04	37,623.08	43,650.91	204,836.72
	177,532.36	149,363.37	157,305.37	150,011.74	139,730.83	773,943.67
Depreciation	35,935.10	34,359.93	32,181.00	29,160.29	27,919.94	159,556.26
	213,467.46	183,723.30	189,486.37	179,172.03	167,650.77	933,499.93
Plant Additions from Current Funds	20,630.33	19,360.99	16,767.59	17,775.06	33,408.40	107,942.37
TOTAL ORDINARY EXPENSE....B	234,097.79	203,084.29	206,253.96	196,947.09	201,059.17	$1,041,442.30
Special Expenditures from Donations	.....	10,433.11	9,705.88	5,129.49	.....	25,268.48
	234,097.79	213,517.40	215,959.84	202,076.58	201,059.17	1,066,710.78
Plant Additions from Donations	.....	23,722.13	44,020.64	.....	.....	67,742.77
TOTAL EXPENSE....Y	$234,097.79	$237,239.53	$259,980.48	$202,076.58	$201,059.17	$1,134,453.55
TOTAL NET (Y−X)	30,449.27	32,526.59	46,543.14	13,089.29	21,908.51	144,516.80
Delete Depreciation	35,935.10	34,359.93	32,181.00	29,160.29	27,919.94	159,556.26
Total Net, less Depreciation	5,485.83	1,833.34	14,362.14	16,071.00	6,011.43	15,039.46
Operating Net (B−A)	79,498.27	63,614.04	70,546.62	51,090.46	59,367.96	324,117.35
Delete Depreciation	35,935.10	34,359.93	32,181.00	29,160.29	27,919.94	159,556.26
Operating Net, Less Depreciation	$ 43,563.17	$ 29,254.11	$ 38,365.62	$ 21,930.17	$ 31,448.02	$ 164,561.09

Marine Biological Laboratory Current Surplus, December 31, 1953

Balance, January 1, 1953			$221,726.86
Add:			
Prior Year Adjustments, Net Recovery		$ 2,293.51	
Transfer from Plant Funds, Net Book Value of Bar Neck Property		19,536.34	
Reserve for Depreciation, 1953, Charged to Plant Funds		35,935.10	57,764.95
			$279,491.81
Deduct:			
Excess of Expense over Income for Year, as		9,818.94	
Payments during Year from Current Funds for Plant Assets:			
Buildings	$ 5,296.64		
Equipment	1,097.49		
Library	14,236.20	20,630.33	30,449.27
Balance, December 31, 1953			$249,042.54

Summary of Bank Balance

Cash Balance, January 1, 1953		
The Hanover Bank	$25,547.59	
Falmouth National Bank	2,547.53	$ 28,095.12
Receipts		563,657.05
		591,752.17
Payments		576,517.30
Cash Balance, December 31, 1953		
Hanover Bank	14,557.93	
Falmouth National Bank	676.94	$ 15,234.87

	Balances Jan. 1, 1953	Received	Paid	Balances Dec. 31, 1953
Allen R. Memhard Fund	$ 86.65	$ 27.50		$ 114.15
Rev. Arsenious Boyer Fund	177.58	148.76	$ 170.00	156.34
Lucretia Crocker Fund	1,192.38	553.16		1,745.54
G. H. A. Clowes Beach Fund	1,000.00			1,000.00
F. R. Lillie Memorial Research Fund	6,393.07	225.00		6,618.07
Retirement Fund	2,897.95			
Bio Club Scholarship Fund	794.75	579.78	1,133.75	240.78
Lalor Foundation Fellowship Fund	976.50	6,000.00	6,117.48	859.02

V. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-sixth session of the Marine Biological Laboratory.

The success of the Marine Biological Laboratory over the past sixty-six years in research and teaching and the development of its plant and facilities surely fulfills the fondest hopes of the founding group. Throughout this period the Laboratory, through its mode of operation, has fostered and encouraged the freest development

of the research ideas of its investigators who themselves, as members of the Corporation, determine its policies.

There has been no suggestion or thought of changing the traditional policies of the Laboratory. In fact, there are many good reasons for continuing along the lines established at the founding of the Laboratory. Probably the most cogent of these is the great need for emphasis on basic research in all fields of science in this country. Fortunately, most of the foundations, including those in the medical sciences and the various governmental agencies controlling research funds, have very enlightened leadership at the policy-making and fund-granting levels. Thus it has been possible for the Marine Biological Laboratory to secure significant support from such sources under grants or contracts. This support is listed under item 1 in this report.

Also, there have been significant contributions listed below from friends of the Laboratory. Included in this is a contribution from the M.B.L. Associates who number 128.

1. Grants, Contracts and Contributions:

These are assuming great importance in the support of the Laboratory. This past year they totaled $108,488.37.

Grants:	
Rockefeller Foundation, general support	$17,500.00
Rockefeller Foundation, special apparatus	5,000.00
National Science Foundation	10,000.00
American Philosophical Society	1,800.00
American Cancer Society	5,350.00
New York Zoological Society	4,000.00
F. R. Lillie Fellowship	1,500.00
National Institute of Health—Studies of Arbacia egg and sperm cells	1,080.00
Contracts:	
Office of Naval Research, studies on marine biology	15,000.00
Office of Naval Research, investigation of environmental factors influencing certain marine biological populations in the Woods Hole area	8,639.50
Office of Naval Research on encephalization during ontogency in *Ameiurus nebulosus*	2,224.00
Office of Naval Research, research in the fundamental properties and functions of nerve tissue by use of the giant axon of the squid	7,695.87
Office of Naval Research, osmoregulation in marine animals	1,000.00
Atomic Energy Comission, research employing radioisotopes in an investigation of the biochemistry of cell nuclei	13,400.00
Atomic Energy Commission, research on the physiology of marine organisms, using radioisotopes	5,400.00
Contributions:	
Mrs. Margaret Meigs	1,000.00
Eli Lilly and Company	5,000.00
Miscellaneous	1,274.00
MBL Associates	1,625.00

2. Plant Improvements:

The great increase in the amount and variety of electrical equipment use in the Laboratory in recent years has resulted in an overloading of some of the electrical circuits. Over the past two years, added lines have been installed and others have been changed to take care of the additional load. Also, new transformers have been installed on the main incoming power lines which are important in the maintenance of constant voltages. Mr. Robert Kahler, Manager of Maintenance, and his staff merit special commendation for their handling of various emergencies which have occasionally occurred in the past due to overloading of lines. With further modifications and changes, it can be confidently anticipated that power failures and voltage fluctuations will be avoided in the future.

3. Operations Analysis:

During the past year, a thorough-going operations analysis of the Laboratory was carried out by Dr. Richard Parmenter who transmitted his final report to the Executive Committee in February (1954). Some of the recommendations made in this report have already been put into effect. Others have been referred to the various appropriate standing committees and to special committees for their consideration. It is anticipated that as a result of this report and the implementation of many of the recommendations, improvements in the operation of the Laboratory will result with continuing information available on the financial operations.

4. Changes in the Dining Hall:

During the past summer, the Dining Hall operated on a self-service basis in a manner that appeared generally satisfactory to the investigators and students. Definite economies resulted. This balanced on a cash basis the financial operation of the Dining Hall which had showed modest deficits in its two preceding summers.

5. Instruction:

Dr. Francis T. Haxo found it advisable to resign as Head of the course in Marine Botany and will be succeeded by Dr. Harold Bold.

The Laboratory was very fortunate in receiving important support from the New York Zoological Society (Mr. Fairfield Osborn, President) for the course in Marine Ecology for the first three years of its operation. This course has been an outstanding success under the leadership of Dr. Bostwick H. Ketchum. It will devolve upon the officers of the Laboratory to seek support elsewhere for the course after 1954.

6. Bar Neck Property:

During the past year, negotiations have continued with the Navy to achieve a settlement for adequate compensation for the Bar Neck Property. Mr. Swope, President of the Corporation, has devoted a great deal of time, thought and energy to this problem. It is hoped that an equitable agreement can be reached in the near future and thus avoid the necessity of long court proceedings.

Respectfully submitted,

PHILIP B. ARMSTRONG,

Director

1. Memorial

Edwin Grant Conklin

1863–1952

E. G. Butler

With the death of Professor Edwin Grant Conklin the biological sciences lost an outstanding leader and the Marine Biological Laboratory one of its earliest and most eminent members. He was born in Waldo, Ohio, November 24, 1863. After a brief illness, he died in Princeton, New Jersey, November 21, 1952.

Most of his youth was spent near Delaware, Ohio, where his father was a country doctor. Here he attended the public schools and later the Ohio Wesleyan University, from which he was graduated in 1885. Although Conklin early became interested in the natural sciences, close second interests, beginning with his undergraduate years, were those in literature and philosophy. With money earned by teaching in a one-room district school during an interval in his college course he began his private library of books in the classics, philosophy, religion, history and science. His deep interest in literature remained with him to the end of his days and his lectures and writings were frequently enlivened by quotations of prose and poetry, much of it committed to memory as a young man.

On graduation from college he took a position at Rust University, Holly Springs, Mississippi, a school for the education of negroes operated by the Methodist Church. There for three years he taught a great variety of subjects, including all of the science offered. It was during this period that he decided to make biology his life work and accordingly he entered the Johns Hopkins University in 1888. Until he enrolled at Johns Hopkins Conklin had had no laboratory training in biology, his knowledge of the subject having come from field collecting, museum work and text-book study. Consequently, during his first year at the Johns Hopkins he took practically all of the undergraduate courses in biology, as well as beginning graduate study under Professor W. K. Brooks. His principal field of study at Johns Hopkins was animal morphology and he received the Ph.D. degree in 1891.

Woods Hole early became Conklin's scientific home. He first came here in the summer of 1890, occupied a research table at the United States Fish Commission, and thus began an association with Woods Hole which was to continue throughout the rest of his life. After two summers at the Fish Commission, he transferred in 1892, on the invitation of Professor Whitman, to the Marine Biological Laboratory. Altogether, his association with Woods Hole as an active research worker covered a span of sixty-two years, a record which I believe is unequaled.

It was during his first summer at Woods Hole that he became interested in the development of the gastropods. He found *Crepidula* abundant and favorable for study and began his work on the embryology and cytology of this form, an investigation which, in one way and another, was to occupy him for many years and to result in numerous important contributions. It was his first summer's research on the early development of *Crepidula*, dealing especially with cell lineage, that formed the subject of his doctoral thesis. It is noteworthy that this was a problem of Conklin's own choice and one which did not at first receive the wholehearted approval of his Johns Hopkins preceptor, Professor Brooks. However, around the turn of the century the method of carefully following the fate of individual cells from the first cleavage of the fertilized egg on through later cleavages to the establishment of parts of the embryo became of prime importance in solving problems concerned with the differential localization of organ-forming substances. In such work Conklin, along with E. B. Wilson and F. R. Lillie, became a leader.

Conklin's research over the years dealt with many different organisms and covered a wide range of subjects in cytology and embryology, but he achieved eminence particularly for his work on the gastropods, the ascidians and *Amphioxus*. He often told of the gratification and delight he experienced when, on examining the eggs of a number of Woods Hole ascidians, he found the naturally-pigmented egg of *Cynthia partita*. As he once wrote, this discovery "modified the whole course and purpose" of his work. It enabled him greatly to extend his earlier studies on the mosaic type of development and the distribution and fate of organ-forming materials. He published many papers on the normal and experimental embryology of *Cynthia* and his great monograph on the *Organization and Cell Lineage of the Ascidian Egg*, published in quarto with 207 figures (60 in color), stands as a landmark of his research.

In connection with his interests in cell lineage and the mosaic type of development, he decided to turn his attention to the early embryology of *Amphioxus*. This work was begun during a period of residence at the Zoological Station in Naples and was continued for many years on material which he had collected there. The results were presented in his papers on the development of *Amphioxus*, including both normal and experimental studies, which greatly extended our knowledge of the embryology of this important form. Despite the comprehensive nature of this investigation, Conklin regarded it as incomplete and those who visited him in his laboratory last summer found him hard at work on his *Amphioxus* material.

Early in his research Conklin began to use experimental methods, especially the application of centrifugal force and changes in temperature and pressure, and to study the cytoplasmic alterations which they produced. More than most biologists he was ever conscious of the relation to one another of the descriptive and the experimental methods and the values of each. His deep interest in experimental zoology is exemplified by the fact that he was one of three zoologists, E. B. Wilson and T. H. Morgan being the others, who were initially responsible for the establishment of the *Journal of Experimental Zoology*. He served on the Editorial Board from the first issue in 1904 until his death.

Of deep concern to Conklin throughout his life were the philosophical and social implications of science. His own research, together with extensive reading, beginning in his youth, in philosophy, religion and social problems, led him to much constructive thinking and the formulation of important concepts dealing with embryology and evolution, evolution and religion, teleology, science and ethics and kindred subjects. He was endowed with an extraordinary ability for penetrating thought on philosophical matters and a remarkable talent for clear and forceful exposition. He was a master in bringing in apt quotations from the great works of science and the classics. The titles and subjects of his books reflect his deep concern with fundamental human problems. *Heredity and Environment in the Development of Men* went through six editions and many printings and was translated into Japanese, French and Russian. His later books, the *Direction of Human Evolution, Freedom and Responsibility, What is Man?*, and *Man: Real and Ideal*, received wide acclaim. In all, he published over 250 works on Embryology, Cytology, Evolution and Education. About half of them deal with evolutionary and philosophical matters. He regarded it as an "inestimable privilege . . . to add to the store of knowledge, to seek truth not only for truth's sake but also for humanity's sake, and to have a part in the greatest work of all time . . . the further progress of the human race."

In his teaching career Conklin was associated with four institutions. On completing his graduate studies at Johns Hopkins, he returned to Ohio Wesleyan University where he served as professor for three years. Then followed two years at Northwestern University and twelve years at the University of Pennsylvania. In 1908, on the invitation of Woodrow Wilson, then president of Princeton University, he joined the Princeton

faculty as Professor of Biology and Chairman of the Department. Here he remained for the rest of his life, becoming Professor Emeritus in 1933.

One cannot include in a memorial such as this an enumeration of the many honors conferred upon him. He was long a member of the National Academy of Sciences and at one time or another served as president of the leading zoological societies of the country. In 1936 he was president of the American Association for the Advancement of Science. He was elected to membership in a number of foreign academies. Honorary degrees of Sc.D. and LL.D. were awarded him by eight universities. Especially intimate was his relation to the American Philosophical Society, in the activities of which he took a leading part for many years. It is particularly noteworthy that in the long history of this organization, the oldest learned society in America, he was the only person to have been twice elected president.

Conklin was one of the old guard of the Marine Biological Laboratory, of the group intimately associated with the early days of the Laboratory under Whitman and with its development through the years. He became a member of the Corporation in 1894 and was a Trustee from 1897 on, becoming Trustee Emeritus in 1934. His counsel in laboratory affairs was frequently sought. As the records show, he served on many standing and special committees and had much to do in solving the problems and shaping the policies of this institution. Few have had a longer or more intimate connection with the Marine Biological Laboratory than he; no one has been more devoted to its welfare. He was a trustee of the Woods Hole Oceanographic Institution, beginning with its establishment in 1930, and last summer was made an Honorary Trustee. With his deep interest in marine biology, he was a leader in the reorganization of the Bermuda Biological Station in 1925 and served as its president for ten years.

A recital of Conklin's achievements in research, his well-deserved eminence as a scholar, his devoted service to many institutions fails to present the complete picture of Conklin as a man. All who knew him came to feel the spell of his warm personal characteristics, to enjoy his brilliance as a conversationalist, his wit, and especially to treasure the stimulation which came from his liberal philosophical and social outlook. His intellectual horizons were broad; he was ever finding new ideas or new and interesting ways of looking at old ideas.

It was Conklin who was called upon to give the principal address at the dedication of this building in 1925. In the course of this address he said: "Our strongest social instincts are for service; the joy of life is in progress; the desire of all men is for immortality through their work." What better words than these can we find to express our appreciation of his life, his work and his contributions to the Marine Biological Laboratory.

Jeremy Babb

1933–1953

Jeremy Babb was born on January 26, 1933, the son of Mr. and Mrs. Glenn Babb of Bedford, Virginia.

Jeremy had just completed his sophomore year as a premedical student at Princeton University and came directly to Woods Hole to work in the Supply Department of the Laboratory. Here he proved himself an able, willing and conscientious worker. He took an active part in the life around him at the Laboratory; his cheerful and friendly personality made him well liked by all who were associated with him.

It was in the early morning hours of July 9, 1953, as he drove to get squid that he met accidental death.

The members of the Corporation of the Marine Biological Laboratory record with deep sorrow the death of Jeremy Babb and they extend heartfelt sympathy to his family.

2. The Staff, 1953

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse

Senior Staff of Investigation

A. P. Mathews, Professor of Biochemistry, *Emeritus*, University of Cincinnati
G. H. Parker, Professor of Zoology, *Emeritus*, Harvard University

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

L. H. Kleinholz, Professor of Biology, Reed College, in charge of course
John H. Lochhead, Associate Professor of Zoology, University of Vermont
Norman A. Meinkoth, Associate Professor of Zoology, Swarthmore College
Grover Stephens, Assistant Professor of Zoology, University of Minnesota
John M. Anderson, Associate Professor of Zoology, Cornell University
Muriel Sandeen, Department of Zoology, Duke University
Sears Crowell, Assistant Professor of Zoology, Indiana University
Howard T. Odum, Assistant Professor of Biology, University of Florida

III. Laboratory Assistants

William E. Dossel, Johns Hopkins University
Elizabeth Paulsen, Rutgers University

EMBRYOLOGY

I. Instructors

S. Meryl Rose, Associate Professor of Zoology, University of Illinois, in charge of course
William E. Berg, Assistant Professor of Zoology, University of California, Berkeley
Mac V. Edds, Jr., Associate Professor of Biology, Brown University
John R. Shaver, Assistant Professor of Zoology, University of Missouri
J. P. Trinkaus, Assistant Professor of Zoology, Yale University
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistants

Lillian M. Youngs, University of North Carolina
Susumu Ito, Western Reserve University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine

ARTHUR K. PARPART, Professor of Biology, Princeton University
ALBERT SZENT-GYORGYI, Director, Institute for Muscle Research, Woods Hole
E. S. GUZMAN BARRON, Associate Professor of Biochemistry, University of Chicago

II. INSTRUCTORS

DANIEL MAZIA, Associate Professor of Zoology, University of California, in charge of course
STEPHEN KUFFLER, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins University Medical School
MAX A. LAUFFER, Professor and Head of Dept. of Biophysics, University of Pittsburgh
ROGER Y. STANIER, Professor of Bacteriology, University of California, Berkeley
H. BURR STEINBACH, Professor of Zoology, University of Minnesota
GEORGE WALD, Professor of Biology, Harvard University
ANDREW SZENT-GYORGYI, Independent Investigator, The Institute for Muscle Research

III. LABORATORY ASSISTANT

PAUL BERNSTEIN, Department of Zoology, Washington University

BOTANY

I. CONSULTANTS

MAXWELL S. DOTY, Associate Professor of Botany, University of Hawaii
WM. RANDOLPH TAYLOR, Professor of Botany, University of Michigan

II. INSTRUCTORS

FRANCIS T. HAXO, Assistant Professor of Marine Botany, Scripps Institution, University of California, in charge of course
PAUL C. SILVA, Instructor in Botany, University of Illinois
RICHARD C. STARR, Instructor in Botany, University of Indiana

III. LABORATORY ASSISTANTS

ANN ALLEN, Indiana University
ROBERT T. WILCE, University of Michigan

IV. COLLECTOR

PETER JENNINGS, Drew University

MARINE ECOLOGY

I. CONSULTANTS

W. C. ALLEE, University of Florida
ALFRED C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

BOSTWICK H. KETCHUM, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
EDWIN T. MOUL, Assistant Professor of Botany, Rutgers University
CHARLES JENNER, Assistant Professor of Zoology, University of North Carolina

III. Assistant

Robert T. Wilce, University of Michigan

THE LABORATORY STAFF, 1953

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
James McInnis, Manager of Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Polly L. Crowell
Mrs. Lila Myers
Nancy Shave
Elizabeth Correllus

LIBRARY

Mary A. Rohan
Elizabeth A. Young
Mary Ann Davis
T. E. Tawell
Albert Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Arthur Callahan
Robert Gunning
John Head
George A. Kahler
Donald B. Lehy
Alton J. Pierce
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Seaver Harlow
Patricia Philpott

SUPPLY DEPARTMENT

Ruth S. Crowell
Milton B. Gray
A. M. Hilton
Walter E. Kahler
Robert Perry
Patricia M. Conway
Geoffrey Lehy
Robert O. Lehy
Carl Schweidenback
James P. Whitcomb
H. S. Wagstaff

BIOLOGICAL BULLETIN

Donald P. Costello, Managing Editor
University of North Carolina, Dept. of Zoology
Chapel Hill, North Carolina
Catherine Henley, Assistant to the Editor

3. Investigators and Students

Independent Investigators, 1953

Allee, Warder Clyde, Head Professor of Biology, University of Florida
Allen, M. J., Assistant Professor of Zoology, University of New Hampshire
Alscher, Ruth Paula, Associate Professor, Manhattanville College of the Sacred Heart
Amberson, William R., Professor and Head of Department, University of Maryland Medical School
Anderson, John Maxwell, Associate Professor of Zoology, Cornell University
Anderson, Robert S., Professor of Physiology, University of South Dakota
Armstrong, Philip B., Professor of Anatomy, State University of New York, College of Medicine at Syracuse
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Bang, Frederick B., Associate Professor of Medicine, Johns Hopkins University School of Medicine
Barlow, Horace B., Instructor, Wilmer Institute, Johns Hopkins Hospital
Bartlett, James H., Professor of Physics, University of Illinois
Berg, William E., Assistant Professor of Zoology, University of California
Berger, Charles A., Chairman, Dept. of Biology, Fordham University
Bernstein, Maurice H., Jr., Staff Member, Carnegie Institution of Washington
Boettiger, Edward G., Associate Professor of Zoology, University of Connecticut
Borei, Hans G., Professor of Zoology, University of Pennsylvania
Boyarsky, Louis L., Associate Professor of Physiology, University of Kentucky
Brown, Frank A., Jr., Professor and Chairman, Dept. of Biological Sciences, Northwestern University
Brownell, Katherine A., Instructor, Ohio State University
Bruner, Joyce A., Research Associate, State University of Iowa
Butler, Elmer G., Professor of Biology, Princeton University
Cantoni, G. L., Associate Professor in Pharmacology, Western Reserve University
Chaet, Alfred B., Research Assistant, University of Pennsylvania
Chase, Aurin M., Associate Professor of Biology, Princeton University
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Claff, C. Lloyd, Associate in Surgery, Harvard Medical School
Clark, Arnold M., Associate Professor of Biology, University of Delaware
Clark, Elliot R., Professor Emeritus of Anatomy, University of Pennsylvania
Clowes, G. H. A., Research Director Emeritus, Lilly Research Laboratories
Clement, A. C., Associate Professor of Biology, Emory University
Cohen, Adolph I., Fellow, National Science Foundation, University of California
Cole, Kenneth S., Technical Director, Naval Medical Research Institute
Colwin, Arthur L., Associate Professor of Biology, Queens College
Colwin, Laura, Professor of Biology, Queens College
Cooperstein, Sherwin J., Assistant Professor of Anatomy, Western Reserve University
Cornman, Ivor, Assistant Research Professor in Anatomy, George Washington University
Costello, Donald P., Kenan Professor in Zoology, University of North Carolina
Crowell, Sears, Assistant Professor of Zoology, Indiana University
Csapo, Arpad I., Staff Member and Lecturer, Carnegie Institution of Washington
deVillafranca, George, Instructor in Zoology, Smith College
Dikshit, Padmakar, Nutrition Research Laboratories, Coonoor, India
Dische, Zacharias, Associate Professor of Biochemistry, Columbia University
Edds, Mac V., Jr., Associate Professor of Biology, Brown University
Eichel, Herbert J., Research Associate, Hahnemann Medical College
Fraenkel, Gottfried S., Professor of Entomology, University of Illinois
Gall, Joseph G., Instructor in Zoology, University of Minnesota
Gasteiger, Edgar I., Research Associate, Massachusetts General Hospital
Goldstein, Lester, Research Associate, University of Pennsylvania
Goodall, Marcus C., Independent Investigator, Institute for Muscle Research
Grant, Philip, Research Fellow, Public Health Service, Columbia University

GREEN, JAMES W., Assistant Professor of Physiology, Rutgers University
GREENE, PETER H., Student and University Fellow, University of Chicago
GRIFFIN, DONALD R., Professor of Zoology, Cornell University
GROSCH, DANIEL S., Associate Professor of Genetics, North Carolina State College
GRUNDFEST, HARRY, Associate Professor in Neurology, College of Physicians and Surgeons
GUTTMAN, RITA, Assistant Professor of Biology, Brooklyn College
GUYSELMAN, J. BRUCE, Instructor in Zoology, Carleton College
HAJDU, STEPHEN, Independent Investigator, The Institute for Muscle Research
HAMER, DOUGLAS, Biochemist and University Research Fellow, Cancer Research Laboratories, Birmingham, England
HAMMOND, WARNER S., Associate Professor of Anatomy, State University of New York, College of Medicine at Syracuse
HARTMAN, F. A., Instructor and Professor, Ohio State University
HARVEY, ETHEL BROWNE, Independent Investigator in Biology, Princeton University
HARVEY, E. NEWTON, Professor of Physiology, Princeton University
HAYASHI, TERU, Associate Professor of Zoology, Columbia University
HAXO, F. T., Assistant Professor of Marine Botany, Scripps Institution of Oceanography
HEILBRUNN, L. V., Professor of Zoology, University of Pennsylvania
HENLEY, CATHERINE, Research Associate, University of North Carolina
HICKSON, ANNA KELTCH, Research Chemist, Lilly Research Laboratories
HILD, WALTHER, Fellow Rockefeller Foundation, University of Colorado
HUNTER, F. R., Professor and Head of Dept. of Physiology, Florida State University
HUXLEY, A. F., Assistant Director of Research, Physiological Laboratory, Cambridge, England
HYMAN, CHESTER, Associate Professor of Physiology, University of Southern California
JACOBS, M. H., Professor of General Physiology, University of Pennsylvania
JENKINS, GEORGE B., Professor Emeritus of Anatomy, George Washington University
JENNER, CHARLES E., Assistant Professor of Zoology, University of North Carolina
JONES, E. RUFFIN, Professor of Biology, University of Florida
KAO, CHIEN-YUAN, Assistant in Pathology, Cornell University Medical College
KALCKAR, HERMAN M., Dept. Head, Institute for Cytophysiology, University of Copenhagen, Denmark
KEMPTON, RUDOLF T., Chairman, Department of Zoology, Vassar College
KIND, CHARLES ALBERT, Assistant Professor of Chemistry, University of Connecticut
KLEINHOLZ, L. H., Professor of Biology, Reed College
KLOTZ, IRVING M., Professor of Chemistry and Biology, Northwestern University
KRAHL, MAURICE E., Associate Professor of Biological Chemistry, Washington University Medical School
KUFFLER, STEPHEN W., Associate Professor, Wilmer Institute, Johns Hopkins Hospital
KUNTZ, ELOISE, Assistant Professor of Physiology, Vassar College
LANSING, ALBERT I., Associate Professor of Anatomy, Washington University Medical School
LAUFFER, MAX A., Professor and Head of Dept. of Biophysics, University of Pittsburgh
LAZAROW, ARNOLD, Associate Professor, Western Reserve University
LEVY, MILTON, Associate Professor of Chemistry, New York University College of Medicine
LOCHHEAD, JOHN H., Professor of Zoology, University of Vermont
LORAND, LASZLO, Associate Department of Physiology, Wayne University College of Medicine
LOVE, WARNER E., Fellow of Johnson Foundation, University of Pennsylvania
MARSLAND, DOUGLAS, Professor of Biology, Washington Square College
MATEYKO, G. M., Fellow, Damon Runyon Mem. Fund, Washington Square College
MAURO, ALEXANDER, Assistant Professor of Physiology, Yale University
MAZIA, DANIEL, Associate Professor of Zoology, University of California
MEINKOTH, NORMAN A., Associate Professor of Biology, Swarthmore College
MERKEL, JOSEPH R., Waksman-Merck Postdoctoral Fellow, Rutgers University
METZ, CHARLES B., Associate Professor of Zoology, University of North Carolina
MIURA, YOSHIAKI, Rockefeller Foundation Fellow, University of Tokyo, Japan
MOORE, GEORGE M., Professor of Zoology, University of New Hampshire
MOORE, JOHN W., Biophysicist, Naval Medical Research Institute
MOUL, EDWIN T., Assistant Professor of Botany, Rutgers University
NACHMANSOHN, DAVID, Associate Professor of Neurology, Columbia University

NANNINGA, LUDO, Independent Investigator, Institute for Muscle Research
NEFF, ROBERT J., Assistant Professor of Biology, Vanderbilt University
NELSON, LEONARD, Instructor of Physiology, University of Nebraska
ODUM, HOWARD THOMAS, Assistant Professor, University of Florida
OSTERHOUT, W. J. V., Member Emeritus, The Rockefeller Institute
PARPART, ARTHUR K., Professor and Chairman, Dept. of Biology, Princeton University
PETTIBONE, MARIAN H., Instructor, University of New Hampshire
PROCTOR, NATHANIEL, Associate Professor of Biology, Morgan State College
PROSSER, C. LADD, Professor of Physiology, University of Illinois
RIESER, PETER, University of Pennsylvania
ROBERTS, JOHN L., Instructor in Physiology, University of Massachusetts
RONKIN, R. R., Assistant Professor of Physiology, University of Delaware
ROOT, WALTER S., Professor of Physiology, Columbia University
ROSE, S. MERYL, Associate Professor of Zoology, University of Illinois
ROTH, JAY S., Assistant Professor of Biochemistry, Hahnemann Medical College
ROYS, CHESTER, Research Associate, Tufts College
RUGH, ROBERTS, Associate Professor of Radiology, Columbia University
SANDEEN, MURIEL I., Instructor in Zoology, Duke University
SCHECHTER, VICTOR, Assistant Professor of Biology, City College of New York
SCHLEYER, WALTER B., Research Associate, Columbia University
SCLUFER, EVELYN, Graduate Student, University of Pennsylvania
SCOTT, ALLAN, Chairman, Department of Biology, Colby College
SCOTT, DWIGHT B. M., Associate in Biochemistry, Children's Hospital of Philadelphia
SCOTT, SISTER FLORENCE MARIE, Professor of Biology, Seton Hill College
SCOTT, GEORGE T., Professor, Oberlin College
SHANES, A. M., Head of Cellular Physiology, National Institute of Mental Health
SARTENAER, PAUL J. M. J., Assistant at Institut Roy. des Sciences Nat., Belgium
SHAVER, JOHN R., Assistant Professor of Zoology, University of Missouri
SHEDLOVSKY, THEODORE, Associate Member, Rockefeller Institute
SILVA, PAUL CLAUDE, Instructor of Botany, University of Illinois
SLIFER, ELEANOR H., Associate Professor of Zoology, State University of Iowa
SOLS, ALBERTO, Research Fellow in Biochemistry, Washington University School of Medicine
SONNENBLICK, B. P., Associate Professor of Biology, Rutgers University
SPEIDEL, CARL C., Professor and Chairman of Anatomy, University of Virginia School of Medicine
SPRATT, NELSON T., Professor of Zoology, University of Minnesota
STANIER, ROGER, Professor of Bacteriology, University of California
STARR, RICHARD C., Assistant Professor of Botany, Indiana University
STEINBACH, H. B., Professor of Zoology, University of Minnesota
STOKEY, ALMA G., Professor Emeritus of Plant Science, Mount Holyoke College
STROMINGER, JACK L., Senior Assistant Surgeon, National Institute of Arthritis and Metabolic Diseases
STUNKARD, HORACE W., Professor of Biology, New York University
SZENT-GYORGYI, ALBERT, Chief Investigator, Institute for Muscle Research
SZENT-GYORGYI, ANDREW G., Independent Investigator, Institute for Muscle Research
SZENT-GYORGYI, EVE, Research Assistant, Institute for Muscle Research
SZENT-GYORGYI, MARTA B., Research Assistant, Institute for Muscle Research
TAYLOR, WM. RANDOLPH, Professor of Botany, University of Michigan
THOMAS, LYELL J., Instructor of Pharmacology, Woman's Medical College of Pennsylvania
TRINKAUS, J. P., Assistant Professor of Zoology, Yale University
TROLL, WALTER, Assistant Director, May Institute for Medical Research
TSUBOI, KENNETH, Chemist, College of Physicians and Surgeons
TRUJI, FREDERIC I., Research Assistant in Biochemistry, Princeton University
VERNBERG, F. JOHN, Instructor of Zoology, Duke University
VILLEE, CLAUDE A., Assistant Professor of Biochemistry, Harvard Medical School
VINCENT, WALTER S., Instructor in Anatomy, New York State University, School of Medicine at Syracuse

WAINIO, WALTER W., Associate Professor of Biochemistry, Rutgers University
WARNER, ROBERT C., Assistant Professor of Chemistry, New York University College of Medicine
WEBB, H. MARGUERITE, Assistant Professor, Goucher College
WHITING, P. W., Professor of Zoology, University of Pennsylvania
WICHTERMAN, RALPH, Professor of Biology, Temple University
WIERCINSKI, FLOYD J., Assistant Professor of Physiology, Hahnemann Medical College
WILBER, CHARLES, Medical Laboratories, Applied Physiology Branch
WILSON, IRWIN B., Assistant Professor of Biochemistry, Columbia University
WILSON, WALTER L., Assistant Professor of Physiology and Biophysics, University of Vermont College of Medicine
WITSCHI, EMIL, Professor of Zoology, State University of Iowa
WRINCH, DOROTHY, Lecturer in Physics, Smith College
ZOTTERMAN, YGVE, Professor, Veterinarske Hogskola, Stockholm, Sweden
ZWEIFACH, BENJAMIN W., Associate Professor of Biology, New York University
ZWILLING, EDGAR, Associate Professor, University of Connecticut

4. LALOR FELLOWS, 1954

DEVILLAFRANCA, GEORGE, Smith College
HAMER, DOUGLAS, Cancer Research Laboratories, Birmingham, England
HUXLEY, A. F., Physiological Laboratory, Cambridge, England
JOHNSON, THOMAS N., Michigan State College
KALCKAR, HERMAN M., University of Copenhagen, Demark
MERKEL, JOSEPH R., Rutgers University
TSUBOI, KENNETH, College of Physicians and Surgeons

Beginning Investigators, 1953

CHANG, JOSEPH J., J. S. DeWitt Fellow in Biology, Princeton University
CHAPMAN, KENT M., Teaching Assistant, University of Minnesota
COHEN, MELVIN J., Graduate Research Zoologist, University of California
COLLIER, JACK R., Graduate Student, University of North Carolina
COUILLARD, PIERRE, Graduate Student, University of Pennsylvania
DECK, J. DAVID, Graduate Student, Princeton University
DUNN, ARNOLD, University of Pennsylvania
EICHHORN, JOHN H., Research Assistant, Ohio State University
GHIRETTI, ANNA, Zoological Station, Naples, Italy
GROSS, PAUL C., Graduate Student, University of Pennsylvania
HEMPLING, HAROLD G., Graduate Student, Princeton University
HERR, EARL B., Graduate Student, University of Delaware
HOWARD, ROBERT S., Assistant Professor of Biological Sciences, University of Delaware
JACOB, MIRIAM, Teaching Assistant, New York University
JOHNSON, THOMAS N., Instructor, Michigan State College
KAYE, ALVIN M., Research Assistant, University of Pennsylvania
KIEBEL, GERALDINE, New York University
LANDAU, JOSEPH V., Damon Runyon Research Fellow, New York University
MARONEY, SAMUEL P., Graduate Student, Duke University
MAYNARD, DONALD M., Teaching Assistant, University of California
MOOS, CARL, Graduate Student, Columbia University
MORRISON, THOMAS H., Graduate Student, Princeton University
PADYKULA, HELEN A., Research Fellow, Harvard Medical School
RAFFERTY, KEEN A., JR., Teaching Assistant, University of Illinois
RAY, DAVID T., Instructor of Zoology, Howard University
ROSENBAUM, LEONARD, Student, University of Delaware

ROSENBERG, ROBERT, Research Fellow, Northwestern University
ROSENBERG, ALBURT M., Graduate Student, University of Pennsylvania
SHANKLIN, DOUGLAS, New York State University College of Medicine at Syracuse
SKOU, J. CHRISTIAN, Assistant Professor, University of Denmark
SMALL, JEAN E., Graduate Student, Brown University
STEPHENS, GROVER C., Instructor in Biology, Brooklyn College
STEPHENSON, WILLIAM K., Graduate Student, University of Minnesota
THRASHER, GEORGE CHARLES, University of Virginia School of Medicine
TUNIK, BERNARD D., Student, Columbia University

Library Readers, 1953

ANGEVINE, JAY B., JR., Graduate Assistant, Cornell University
BAKER, HOWARD D., Associate Professor, Florida State University
BAUCHAU, ADRIAN G., Charge de Cours in Zoology, Facultás Universitaires, Namur, Belgium
BLOCH, ROBERT, Research Associate, University of Pennsylvania
BODANSKY, OSCAR, Chief of Clinical Biochemistry, Sloan Kettering Institute
COHEN, SEYMOUR S., Associate Professor of Physiol. Chem., University of Pennsylvania, Children's Hospital
DILLER, IRENE COREY, Associate Member of Institute, Institute for Cancer Research
DIXON, FRANK J., Professor and Chairman, Dept. of Pathology, University of Pittsburgh School of Medicine
DUBOIS, EUGENE F., Emeritus Professor of Physiology, Cornell University Medical College
GABRIEL, MORDECAI L., Assistant Professor, Brooklyn College
GABRIELI, ELEMER, Research Fellow, Yale Medical School
GAFFRON, HANS, Professor of Biochemistry, University of Chicago
GRUENBERG, BENJAMIN C., Consultant, 100 Central Park South, New York 19
HENSHAW, PAUL S., Director of Research, Planned Parenthood Federation of America
JONES, SARAH R., Instructor of Zoology, Connecticut College
KABAT, ELVIN A., Professor of Microbiology, College of Physicians and Surgeons
KARRER, HEINZ E., Johns Hopkins University School of Hygiene and Public Health
KARUSH, FRED, Assistant Professor of Immunology, University of Pennsylvania
KINDRED, JAMES E., Professor of Anatomy, University of Virginia
KINERSLY, THORN, Research Fellow, Yale University School of Medicine
KRAMER, MOLLIE P., Bibliographer, American Meteorological Society
LIANG, HSU-MU, Fellow of Cancer Research, Washington University
LOVE, LOIS CODER, Chemical Biological Coordination Center, National Research Council
LUCKE, BALDUIN, Professor of Pathology, University of Pennsylvania
MCDONALD, SISTER ELIZABETH SETON, Chairman, Dept. of Biology, College of Mount St. Joseph
MEMHARD, ALLEN R., 18 Crescent Road, Riverside, Connecticut
MORRISON, DAVID, Woods Hole, Mass.
NAMIAS, JEROME, Chief, Extended Forecast Section, U. S. Weather Bureau
ORTIZ, EVELINA, Instructor in Zoology, University of Chicago
RACKER, EFRAIM, Associate Professor of Biochemistry, Yale University
REINER, JOHN M., Assistant Professor of Biochemistry, Columbia University
ROSS, VICTOR, Research Associate, College of Physicians and Surgeons
SCHWARTZ, MARTIN, University Club, Madison, Wisconsin
SCHWARTZMAN, GREGORY, Director, Dept. of Microbiology, Mount Sinai Hospital
SCOTT, THOMAS F. M., Director of Research, Childrens Hospital of Philadelphia
STEIGMAN, ALEX J., Professor of Child Health, University of Louisville Medical School
STERN, KURT G., Adjunct Professor of Biochemistry, Polytechnic Institute of Brooklyn
SULKIN, S. EDWARD, Professor and Chairman, Dept. of Bacteriology, Southwestern Medical School
TONDO, C. V., National Research Council of Brazil
TROTTER, MILDRED, Professor of Gross Anatomy, Washington University
TSAI, TSULI, Fellow of Cancer Research, Washington University Medical School
TYLER, ALBERT, Professor of Embryology, California Institute of Technology

WILHELM, J. O., Director, Research Council of Ontario, Toronto, Canada
WOLLMAR, RICHARD, Technician, Seaplant Chemical Corporation
WYMENGA, HARRY G., Research Chemist, N. V. Organon, Oss, Holland
ZINN, DONALD J., University of Rhode Island

Research Assistants, 1953

ALLEN, MARJORY ANN, Indiana University
BAKER, GERALDINE M., Western Reserve University
BARNES, LOY J., University of Missouri
BENNETT, MIRIAM F., Northwestern University
BERMAN, MONES, Sloan Kettering Institute
BERNSTEIN, PAUL W., Washington University
BLANKENHORN, BRIGITTE, University of Maryland Medical School
BOOTH, ELIZABETH A., Columbia University
BORENFREUND, ELLEN, Columbia University
CHALFIN, DAVID, Princeton University
DETERRA, NOEL, Barnard College, Columbia University
DOSSEL, WILLIAM E., Johns Hopkins University
DRAKE, JOHN W., Yale University
FOX, HOWARD A., Colby College
FROST, JAMES L., Princeton University
GINTER, EILEEN L., Hahnemann Medical College
GLASSER, RICHARD L., University of Maryland Medical School
GNADE, ALLA, University of Pennsylvania
GRAVES, ROBERT C., Northwestern University
HINES, MARGARET N., Northwestern University
HOHNS, ELSIE D., New York University
ITO, SUSUMU, Western Reserve University
LACHANCE, LEO EMERY, North Carolina State College
LARIS, PHILIP C., Princeton University
LAUFER, WILMA, Tufts College
LECRONE, PATRICIA A., University of Delaware
LORIMER, ISABELLE, New York University
MCLAUGHLIN, JEAN, The Institute for Muscle Research
MAWE, RICHARD C., Princeton University
METZ, DELILAH B., New York University
PAULSEN, ELIZABETH, University of Vermont
PHILLPOTT, DELBERT, The Institute for Muscle Research
REICH, PETER, Harvard Medical School
RIMMLER, LUDWIG J., Syracuse University
RODGERS, FRANCES F., University of Pennsylvania
ROSENBLUTH, RAJA, Columbia University
SCHWARZ, NANCY L., University of Illinois
SERES, JOEL L., University of Delaware
STEINBERG, MALCOLM S., University of Minnesota
STONE, NOMI, Barnard College
SULLIVAN, ROBERT L., North Carolina State College
SUSSMAN, KARL E., University of Maryland Medical School
VAN BERGEIJK, WILLEM, State University of Iowa
VOORHEES, DAVID B., Yale University
WALTERS, PATRICIA, Lilly Research Laboratories
WATT, DONALD J., Columbia University
WESTFALL, JANE ANNE, University of North Carolina
WILCE, ROBERT T., University of Michigan
WYTTENBACH, CHARLES R., Indiana University
YOUNGS, LILLIAN M., University of North Carolina
ZIMMERMAN, ARTHUR M., New York University

Students, 1953

BOTANY

Bowes, Rev. David, Catholic University
Hillis, Llewellya, University of Michigan
Kanwisher, Joan, Woods Hole, Mass.
Marshall, Harold G., Western Reserve University
Dowling, John A., Harvard College
Noe, Frederick, Drew University
Rudolph, Emanuel, Washington University
Smith, Charlotte A., Vassar College
Stephenson, Phyllis C., University of Illinois
Stibritz, Mary G., Earlham College
Young, Richard S., University of North Carolina

ECOLOGY

Angevine, Emeline Midgett, Cornell University
Bond, Richard R., University of Wisconsin
Bronsweig, Ruth D., Bryn Mawr College
Greenburg, Michael J., Cornell University
Kuenzler, Edward J., Jr., University of Georgia
Perrotta, Carmie A., University of Wisconsin
Schreiman, David E., 107 Gifford Avenue, Jersey City, N. J.
Shaw, Evelyn S., American Museum of Natural History

EMBRYOLOGY

Ambellan, Elizabeth H., Columbia University
Beskind, Harry, Queens College
Borysko, Emil, Johns Hopkins University
Boulas, Stanley H., Brown University
Brailsford, Elizabeth S., Mt. Holyoke College
Clugston, Helen V., Oberlin College
Curley, Ellen L., Wellesley College
Dashman, Theodore, University of Illinois
Denny, David, Dillard University
Doolin, Paul F., University of Illinois
Duncan, James T., Wabash College
Erickson, Joan, Radcliffe College
Gallagher, John C., Yale University
Grabowski, Casimer T., Johns Hopkins University
Hegan, Nancy A., Pennsylvania College for Women
Hilfer, Saul Robert, Queens College
Hodge, Mary H., Simmons College
Jeffries, William B., University of North Carolina
LeTourneau, David J., Wesleyan University
McKinnell, Robert G., Springfield 4, Missouri
McMurray, Virginia M., State University of Iowa
Merrill, Arthur S., Harvard University
Milkman, Roger D., Harvard University
Park, Helen D., George Washington University
Rudin, Robert, University of North Carolina
Spiegelman, Irvin Morton, Amherst College
Swartz, Frank J., Western Reserve University
Telfer, William H., Harvard University
Tucker, Rev. Eugene L., Loyola College
Wessells, Norman K., Yale University

PHYSIOLOGY

BAKEMEIER, RICHARD F., University of Rochester
BEHRMAN, EDWARD T., University of California
BIBRING, THOMAS, Stanford University
BIRKS, RICHARD I., McGill University
BRILL, ARTHUR S., University of Pennsylvania
CASCARANO, JOSEPH, New York University
CHILD, FRANK M., Amherst College
FEBVRE, HENRI, The Rockefeller Institute
GELLER, DAVID M., Harvard University
GONZALEZ, ELSA L., Columbia University
HAGGIS, ALEX J., University of Rochester
HARRIS, JOSEPH D., Purdue University
HARVEY, HARRIET R., University of Oklahoma
KOWAL, JEROME, Johns Hopkins Medical School
LAMBERT, FRANCIS L., Harvard University
LEVENTHAL, DAVID E., University of Illinois
LITMAN, ROSE M., Indiana University
MANN, JAY D., Brooklyn College
MILLS, KENNETH S., University of Texas
OLSON, JOHN M., University of Pennsylvania
POTTER, DAVID D., Harvard University
RAPOPORT, STANLEY I., Princeton University
RIECK, ALVIN F., Marquette University
ROEDER, MARTIN, University of North Carolina
ROTER, ELLEN CELIA, University of Illinois
ROTHSCHILD, HANNA A., University of Sao Paulo, Brazil
SCHNEIDERMAN, HOWARD A., Cornell University
SOLANO, SISTER FRANCIS, Nazareth College
STRAUS, MARK, 1901 Bedford Avenue, Brooklyn, N. Y.
THEOPHILUS, VICTORIA, Vassar College
WOLBACH, ROBERT A., Cornell University School of Medicine

INVERTEBRATE ZOOLOGY

ASHTON, FRANCIS T., Swarthmore College
ATZ, JAMES W., New York Zoological Society
BARSA, MARY C., Fordham University
BENDIX, SELINA, University of California
BIERMAN, SHEILA, Cornell University
BROWN, PAUL L., University of Illinois
CORY, ROBERT L., University of Delaware
CUMINGS, EDWIN H., DePauw University
CURRY, GEORGE M., Harvard University
DOWLING, MARY C., Seton Hill College
DRAKE, JOHN W., Yale University
EISENMAN, JOSEPH, City College of New York
FRASER, ELIZABETH F., Bedford College, London, England
FRASER, JEAN F., Bedford College, London, England
FROST, DAVID, New York University
GREEN, DONALD M., Oberlin College
GREEN, PAUL B., University of Pennsylvania
HADLEY, DORIS S., George Washington University
HAGEN, LYNETTE M., Brothers College of Drew University
HARTSHORNE, JAMES M., Cornell University
HARVEY, WILLIAM R., Harvard University
HAUBRICH, ROBERT R., University of Florida
HEAFITZ, MORTON H., Dartmouth College
HICKOK, JOHN F., Cate School, California

LAPPANO, ELEANOR R., Fordham University
LINDER, HARRIS J., Cornell University
MARKO, ANITA R., Adelphi and Hofstra Colleges
MCDANIEL, HARRIET C., Brooklyn College
MCKERNAN, SALLY A., University of Kansas
MCNAMARA, DORRIS, New York University
MESSINGER, ELI CHARLES, Lafayette College
MIZELL, MERLE, University of Illinois
MOFFATT, JANE L., George Washington University
PASSAGLIA, MARTIN A., St. Louis University
PATTON, WENDELL K., Hamilton College
PIERRO, LOUIS, Marquette University
PRYOR, CARLON W., University of Kansas
REED, ROBERT R., Purdue University
RENNER, JOHN H., Dartmouth College
RICHARDS, MARIE L., Maryville College
ROBERTS, JANE C., University of Massachusetts
ROSLANSKY, JOHN D., University of California
ROWE, EDWARD C., Wesleyan University
RUNYON, MIRIAM, Vassar College
SARGENT, LYDIA M., Knox College
SCHLICK, ROBERTA A., Knox College
SCHLUETER, EDGAR A., University of Wisconsin
SENTERFIT, LAURENCE B., Johns Hopkins Hospital
SHRINER, JOAN, Mount Holyoke College
SKINNER, DOROTHY M., Tufts College
STEVENSON, JOSEPH R., Oberlin College
SULLIVAN, ROBERT L., North Carolina State College
VANLAER, HELEN R., Johns Hopkins University
WARWICK, ANNE CHARLOTTE, Johns Hopkins University
WENDT, RICHARD H., University of Rochester

5. TABULAR VIEW OF ATTENDANCE, 1949–1953

	1949	*1950*	*1951*	*1952*	*1953*
INVESTIGATORS—TOTAL	344	338	303	300	310
Independent	193	198	186	172	176
Under Instruction	52	43	28	38	37
Library Readers	55	48	37	49	46
Research Assistants	44	49	52	47	51
STUDENTS—Total	128	126	124	123	136
Zoology	55	55	55	55	55
Embryology	31	29	27	23	30
Physiology	27	27	29	27	31
Botany	15	13	13	11	11
Ecology				7	9
TOTAL ATTENDANCE	472	444	427	423	446
Less persons registered as both students & investigators	2			2	
	470	444	427	421	446
INSTITUTIONS REPRESENTED—Total	155	156	158	149	155
By Investigators	114	114	115	92	90
By Students	68	67	43	57	65
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	1	2	1	1	
By Students				3	1
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	6	6	8	7	15
By Students	3	2	3	2	6

6. Cooperating and Subscribing Institutions, 1953

Cooperating Institutions

Amherst College
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Carnegie Embryological Laboratory
Children's Hospital of Philadelphia
Colby College
College of Mt. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Cornell University
Drew University
Duke University
Elmira College
Emory University
Fordham University
George Washington University
Hahnemann Medical School
Harvard University
Harvard University Medical School
Eli Lilly and Company
Marquette University
Massachusetts General Hospital
Morgan State College
Mount Holyoke College
National Institute of Arthritis and Metabolic Diseases
National Science Foundation
New York University College of Medicine
New York University, Heights
New York University, Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oak Ridge Institute for Nuclear Studies
Oberlin College
Princeton University
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Seaplant Chemical Corporation
Sloan Kettering Institute
Southwestern Medical College
State University of Iowa
State University of New York, College of Medicine at Syracuse
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Delaware
University of Illinois
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of New Hampshire
University of North Carolina
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Vermont Medical School
University of Virginia
University of Wisconsin
Vassar College
Washington University School of Medicine
Wellesley College
Wesleyan University
Western Reserve University School of Medicine
Yale University

Subscribing Institutions

American Bureau of Medical Aid to China
Belgian American Educational Foundation
City College of New York
Cleveland Audubon Society
Dillar University
Hamilton College
Indiana University
Loyola College
May Institute for Medical Research
Mount Sinai Hospital
Ohio State College
Pennsylvania College for Women
Radcliffe College
University of California
University of Kentucky
University of Louisville Medical School
University of Mississippi
University of Nebraska
University of Pittsburgh
Vanderbilt University
Wabash College
Washington University
Women's Medical College of Pennsylvania

7. Evening Lectures, 1953

July 3
Hermann Kalckar ..... "Nucleotides, their synthesis and their functions."

July 10
Horace W. Stunkard ..... "Parasitism, a phase of marine biology."

July 17
E. S. G. Barron ..... "The importance of sulfhydryl groups in biology and medicine."

July 24
H. Keffer Hartline ..... "Electrical activity of visual receptors and optic nerve fibers."

July 31
Robert Briggs ..... "The problem of nuclear differentiation in embryonic development."

August 7
P. F. Scholander ..... "Homiothermism in relation to climate."

August 10
A. F. Huxley ..... "Muscle fibers under the microscope."

August 14
Ernest Pollard ..... "The physics of viruses."

August 21
Leigh E. Chadwick ..... "Insect cholinesterases."

8. Tuesday Evening Seminars, 1953

June 20
Dorothy Wrinch ..... "New evidence regarding the structure of ribonuclease."
W. S. Vincent ..... "An approach to the problem of DNA constancy."
Daniel Mazia ..... "Chemical and electron microscopic observations on the isolated mitotic apparaus."

July 7
Warner Hammond ..... "The neuronal derivatives of the neural crest in the chick."
Sears Crowell ..... "The regression-replacement cycle of hydranths in *Obelia* and *Campanularia*."
Motion Picture: ..... F. S. Hammett's time lapse study of the development and regression of hydranths of Obelia.

July 14
Harold G. Hempling ..... "Potassium and sodium movements in rabbit polymorphonuclear leukocytes."
L. Lorand ..... "Clotting of fibrinogen as a problem of protein chemistry."
Max A. Lauffer, A. Buzzell and D. Trkula ..... "Thermal inactivation of a bacterium-bacteriophage complex at various stages of development."

July 21

IRWIN B. WILSON ..... "Some features of the proteins associated with the generation of bioelectricity."

MARIO ALTAMIRANO ..... "The relation between the chemical structure of some compounds and their effect upon bioelectric phenomena."

Y. ZOTTERMAN ..... "The effect of acetylcholine and related compounds on sensory organs."

July 28

JAMES W. GREEN and F. X. WAZETER .. "Glycolysis and potassium loss in rabbit red cells exposed to ethyl and propyl carbonate."

F. R. HUNTER and ALICE S. BAKER .... "A study of the oxidative activity of rabbit reticulocytes."

GEORGE T. SCOTT and HUGH HAYWARD .. "Metabolic factors influencing the distribution of sodium and potassium ions in the green alga *Ulva lactuca*."

August 4

STEPHEN HAJDU ..... "On the mechanism of staircase and contracture in ventricular muscle."

C. LADD PROSSER ..... "Conduction in non-striated muscles."

ABRAHAM M. SHANES ..... "Structural and *in vitro* diffusion characteristics of intact and 'desheathed' sciatic nerves from the toad (*B. merinus*) and the bullfrog (*R. catesbiana*)."

August 11

JAY S. ROTH ..... "Some observations on the ribonuclease activity of separated cell particulates."

WALTER TROLL and SOL SHERRY ..... "Synthetic substrates for fibrinolysin (plasmin) and thrombin."

August 18

MALCOLM STEINBERG ..... "Studies on the mechanism of physiological dominance in Tubularia."

WALTHER HILD ..... "Elaboration of so-called posterior lobe hormone in the hypothalamus."

Y. ZOTTERMAN ..... "Primary polydipsia in goats (motion picture)."

9. MEMBERS OF THE CORPORATION, 1953

1. LIFE MEMBERS

BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, New York.
BILLINGS, MR. R. C., 66 Franklin Street, Boston, Massachusetts.
BUDINGTON, DR. R. A., Box 954, Winter Park, Florida.
BRODIE, MR. DONALD M., 522 Fifth Avenue, New York 18, New York.
CALVERT, DR. PHILIP P., University of Pennsylvania, Philadelphia, Pennsylvania.
COLE, DR. LEON J., College of Agriculture, Madison, Wisconsin.
COWDRY, DR. E. V., Washington University, St. Louis, Missouri.
DUNGAY, DR. NEIL S., Carleton College, Northfield, Minnesota.
GOLDFARB, DR. A. J., College of the City of New York, New York City, New York.
JACKSON, MR. CHARLES C., 24 Congress Street, Boston, Massachusetts.

JACKSON, MISS M. C., 88 Marlboro Street, Boston, Massachusetts.
KING, MR. CHARLES A.
KING, DR. HELEN D., Wistar Inst. of Anatomy and Biology, Philadelphia, Pennsylvania.
LEWIS, PROF. W. H., Johns Hopkins University, Baltimore, Maryland.
LOWTHER, DR. FLORENCE DEL., Barnard College, New York City, New York.
MACNAUGHT, MR. FRANK M., Woods Hole, Massachusetts.
MEANS, DR. J. H., 15 Chestnut Street, Boston, Massachusetts.
MOORE, DR. GEORGE T., Missouri Botanical Gardens, St. Louis, Missouri.
MOORE, DR. J. PERCY, University of Pennsylvania, Philadelphia, Pennsylvania.
NOYES, MISS EVA J.
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VI. REPORT OF THE LIBRARIAN

1953

During 1953, the number of journals currently received totalled 1503 (67 new). There were 461 (14 new) Marine Biological Laboratory subscriptions; 600 (21 new) exchanges and 163 (15 new) gifts; 73 (6 new) were Woods Hole Oceanographic Institution subscriptions; 173 (5 new) were exchanges and 33 (6 new) were gifts.

The Marine Biological Laboratory purchased 39 books, received 51 complimentary copies (7 from authors and 44 from publishers) and 14 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 23 titles and received 7 gifts. The total number of books placed on the shelves amounted to 134. The Marine Biological Laboratory completed by purchase 7 journal sets and partially completed 9 sets. The Woods Hole Oceanographic Institution completed 3 back sets and partially completed 4 sets. Volumes and numbers were received by gift and by exchange which partially completed 9 sets.

The reprint collection was increased by 5643 papers, 2464 being of current issue.

Forty-seven titles were borrowed on inter-library loan and 125 were sent to out-of-town libraries. The sale of duplicate material contributed $263.45 to the Laboratory's general fund. The microfilm service has not as yet been resumed.

The Library gratefully acknowledges the following gifts: from Dr. Paul S. Galtsoff, his card bibliography of references pertaining to the "Gulf of Mexico—Its Origin, Waters and Marine Life," as well as numbers and volumes which further complete 10 Russian titles; a set of the Josiah Macy Foundation publications from the Foundation; a current subscription to "Scientific American" from Dr. Ross G. Harrison, Jr.; the early volumes of "Nucleonics" from Mr. Jack Hagstrom; and reprint collections from Dr. and Mrs. P. W. Whiting, Smith College Library, Dr. L. J. Milne, Dr. Ursula M. Höber, and the U. S. Army Chemical Center in Maryland.

At the end of the year, the Library contained 63,276 bound volumes and 188,584 reprints.

The Librarian wishes to express her appreciation to Dr. Wm. Randolph Taylor for the help and advice so willingly given during his term as Chairman of the Library Committee.

Respectfully submitted,
DEBORAH L. HARLOW,
Librarian.

PHASE-CONTRAST AND ELECTRON MICROSCOPE STUDIES ON THE NEBENKERN, A MITOCHONDRIAL BODY IN THE SPERMATIDS OF THE GRASSHOPPER

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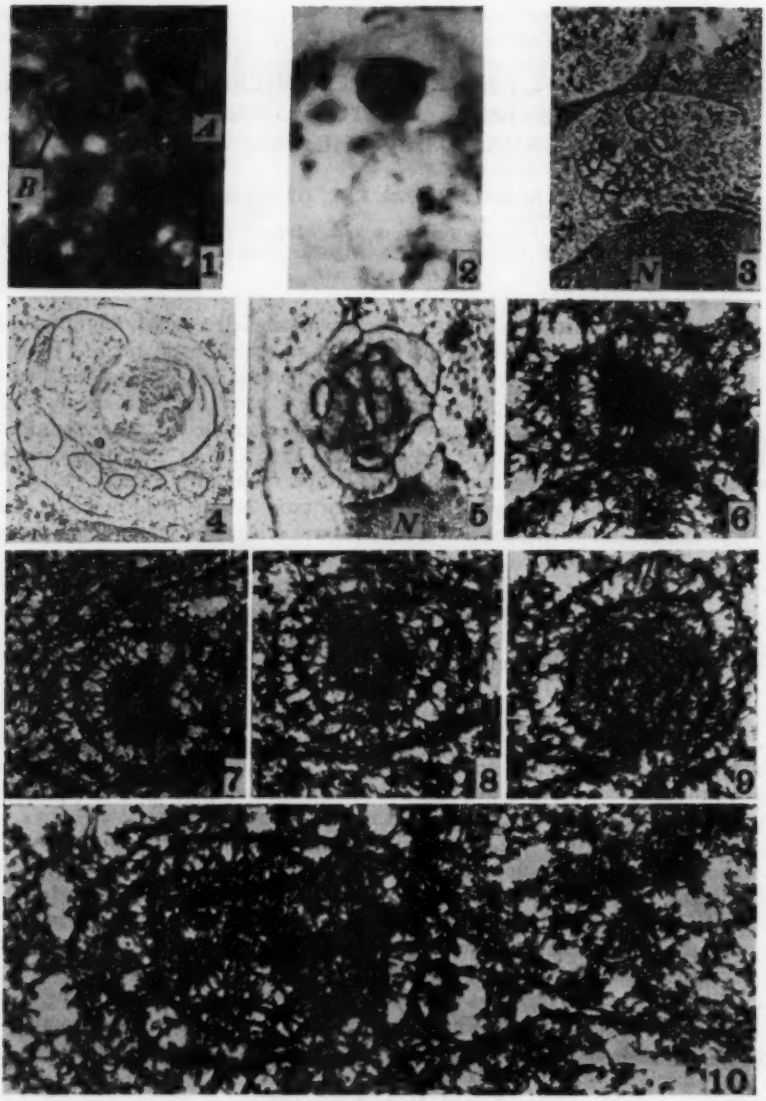
Attempts to demonstrate a structure within mitochondria which might be correlated with their function have been the object of several investigations: (1) In the germ cells of certain insects the filamentous mitochondria are composed of two distinct parts, an outer chromophilic and an inner chromophobic (Gatenby, 1917; Bowen, 1922). (2) In electron micrographs certain mitochondria show areas of unequal electron density (Claude and Fullman, 1945). (3) Sometimes the particles appear to be held together within a lattice framework (Pease, Baker and Warren, 1950). (4) Other mitochondria show cross striations (Pease and Baker, 1950), while still others possess a system of internal ridges, or cristae, that protrude perpendicularly from the inside surface of the membrane toward the interior of the mitochondrion (Palade, 1952, 1953). Similar structures have been observed to extend across the mitochondria and to be composed of double membranes (Sjöstrand, 1953). (5) In contrast to the above, certain other mitochondria display longitudinally oriented internal filaments or lamellae (Glimstedt and Lagerstedt, 1953; Beams and Tahmisian, 1954). Mitochondria of this type show birefringence in their longitudinal axis (Monné, 1948). In addition, variation in mitochondrial form, size and structure has been shown to occur in cells of animals exposed to pathological or experimental conditions. The demonstration of a limiting membrane at the surface of the mitochondria by Dalton *et al.* (1949) has, for the most part, been confirmed (Palade, 1952; Sjöstrand, 1953 and others).

Since the nebenkern in the spermatids of certain animals has been shown to be a mitochondrial body, *i.e.*, composed of fused mitochondria (von la Valette St. George, 1886; Meves, 1900; Lewis and Robertson, 1916; Payne, 1916, 1926; Gatenby, 1917; Bowen, 1922; Pollister, 1930; Johnson, 1931 and others), it seemed of interest to attempt by aid of the electron microscope an analysis of its ultramicroscopic structure and to compare the structure thus revealed with that cited above for other mitochondria. Limited observations have also been made on the nebenkern of living cells by aid of the phase-contrast microscope, and the structure thus revealed compared to that of comparable stages described for it in fixed and stained preparations.

Material and Methods

The material for this study consists of the testes of grasshopper nymphs, *Melanoplus differentialis*. After removing the testis from the body, it was placed in Bělař solution and dissected free of fat and connective tissue. About three tubules were then placed in a few drops of Bělař solution in the lower half of a roto-compressor



All Figures in Plates I to III (except Figs. 1 and 2) are electron micrographs. Figures 1 and 2 are phase-contrast microscope pictures of living cells.

PLATE I

FIGURE 1. Phase-contrast microscope picture of two nebenkern. The one at A is in a relatively early stage of development and shows a comparatively dense core surrounded by a vacuolar layer and a limiting membrane or lamella. The one at B is a mature stage and shows

where their tips were cut to allow the cells of the tubules to be displaced into the surrounding fluid. The top of the roto-compressor was then placed in position and tightened until the cells showed slight compression when viewed through the Leitz phase-contrast microscope.

The fixatives used for electron microscope studies were Champy's 2% osmic acid, and buffered 2% osmic acid (pH 7.25). Tubules from fixed testes were embedded in methacrylate, sectioned at 0.1 μ and studied by aid of an R. C. A. model E. M. U. electron microscope.

Results

Phase-contrast microscope observations

Preliminary observations on the structure of the nebenkern in different stages of development with the phase-contrast microscope showed it to be essentially the same as that seen in fixed and stained preparations. For this reason, an extensive study on the structure of the nebenkern by this method was not attempted. However, we have included two figures of the phase-contrast microscope part of this study, each of which resembles closely similar stages figured by Bowen (1922) and others. Figure 1A, according to Bowen (1922), is a relatively early stage in the differentiation of the nebenkern. It will be noted that at this stage a light area is evident near the periphery, which is presumably being differentiated by a vacuolization process from the central opaque region. This process continues until a fully differentiated nebenkern with concentric lamella of about 0.50 μ in width and separated by inter-lamellar spaces is formed (Fig. 1B). Here also is seen a plate or filament arranged at right angles to the concentric lamellae; the latter are probably associated with the division mechanism occurring in the nebenkern of older spermatids. Figure 2 shows a vacuolated condition of the nebenkern which is believed to be a relatively late stage in its existence.

As will be noted below, it is difficult to correlate all the structure of the nebenkern seen with the phase-contrast and light microscopes with that observed in electron micrographs.

Electron microscope observations

Out of approximately 150 electron micrographs of spermatids showing nebenkern, we have attempted to arrange in succession certain ones which seem to show pro-

a series of concentric lamellae alternating with layers of matrix. A relatively dense membrane extends across its axis. × 1000.

Figure 2. Phase-contrast picture of a vacuolated nebenkern. × 1000.

Figure 3. Group of mitochondria (M) that are apparently fusing in the initial stages of the formation of the nebenkern. The nucleus is shown at N. Buffered 2% osmic acid fixation. × 5000.

Figures 4 and 5. Early stages in the formation of the nebenkern showing more extensive fusion of vacuoles and the initial stages in formation of the concentric lamellae. In Figure 5 the nucleus is shown at N. Buffered 2% osmic acid fixation. × 5000.

Figure 6. Section through the peripheral region and across the long axis of a mature nebenkern. Champy's fixation. × 5000.

Figures 7 and 8. Sections through consecutive deeper levels of the mature nebenkern revealing its internal structure. Champy's fixation. × 10,000.

Figure 9. A mature stage of nebenkern showing 4 or 5 concentric lamellae. Champy's fixation. × 10,000.

Figure 10. Obliquely sectioned nebenkern. Some of the lamellae in upper half of figure seem double. Champy's fixation. × 17,000.

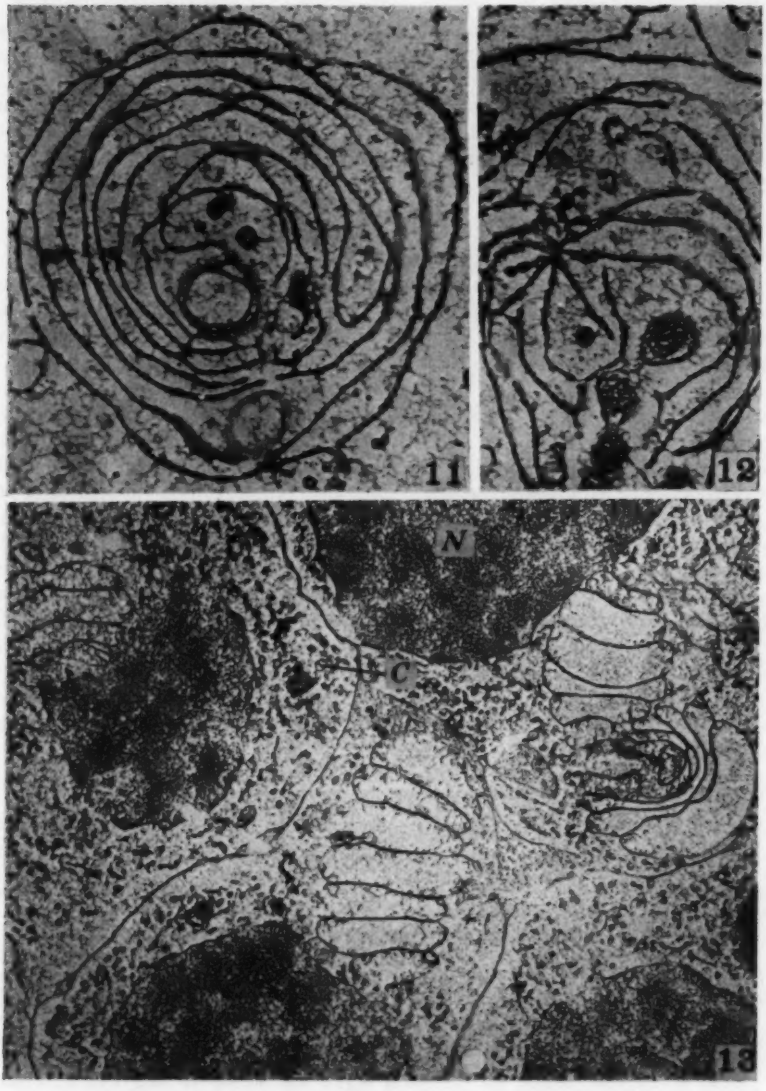


PLATE II

FIGURE 11. Cross section of mature nebenkern. Here the lamellae measure about 400 Å in thickness. The interlamellar matrix shows no special structure. Buffered 2% osmic acid fixation. × 16,500.

gressive stages in structural differentiation, the peak of which is followed by dedifferentiation in the transforming spermatid. Such an arrangement is not an easy task, even when studied in light microscope preparations (Bowen, 1922), and it becomes an even more difficult one when studied at the ultramicroscopic level.

Immediately following division of the second spermatocyte the somewhat swollen and vesicular mitochondria collect at one side of the nucleus in the newly formed spermatids (Fig. 3M). As development proceeds, they seem to fuse (Figs. 4, 5, and 18A), giving rise to elongated vesicles, the surfaces of which appear to form the dense concentric laminated structures; the remaining part gives rise to the less dense inter-lamellar matrix (Figs. 9, 10, 11, and 12).

As will be noted below, when completely differentiated the nebenkern is composed of a series of concentric lamellae separated by inter-lamellar matrix. Figures 6 to 10 seem to show a series of transverse sections at different depths through the completely differentiated nebenkern. Figure 6 appears to represent a section through the peripheral region and across the longitudinal axis. If the lamellae are numbered from the outside inward, Figure 6 represents a section in which the knife passed across the first, or outer, lamella and through, or just above, the second lamella. Similar, but deeper, sections are noted in Figures 7 and 8 where they are through the level of the third and fourth lamellae, respectively. When a more central section is made through the nebenkern, 6 to 8 concentric lamellae are often evident (Figs. 9 to 11). In Figure 9 the section is through the longitudinal axis of the nebenkern; it is in this axis that it will eventually divide (Figs. 14, 15) and elongate (Fig. 16), giving rise to part of the structure in the middle piece of the sperm. The lamellae of the nebenkern measure about 400 Å in thickness and the inter-lamellar spaces about 1800 to 3000 Å in thickness. Figure 10 is an oblique section through the nebenkern and is interesting because some of its lamellae appear to be composed of double membranes. When viewed in cross section the concentric lamellae of the nebenkern are approximately eight in number (Fig. 11). Little structure is observed within the inter-lamellar matrix which could be used to differentiate it from the surrounding cytoplasm. A section through one pole of the nebenkern is seen in Figure 12, while Figure 13 shows sections through the sides of what seem to be developing stages. The scattered vesicular bodies in the cytoplasm of these cells are probably diffuse basophilic substance or what Porter (1953) has called endoplasmic reticulum (Fig. 13C).

In older spermatids, those in the initial stages of transformation into spermatozoa, the nebenkern divides into two parts (Figs. 14, 15, and 16). At this time it loses its lamellar structure, becomes somewhat vesicular, and elongates into the middle piece of the developing sperm (Fig. 16). A few electron-opaque bodies are still evident within the halves of the nebenkern but the lamellae have disappeared, leaving a comparatively structureless matrix. A centriole may be observed here at

FIGURE 12. Section near one pole of nebenkern, revealing the arrangement of lamellae. Buffered 2% osmic acid. × 17,000.

FIGURE 13. Longitudinal section through side of developing nebenkern revealing outer lamellae. The nucleus is shown at N and the endoplasmic reticulum at C. Buffered 2% osmic acid fixation. × 5000.

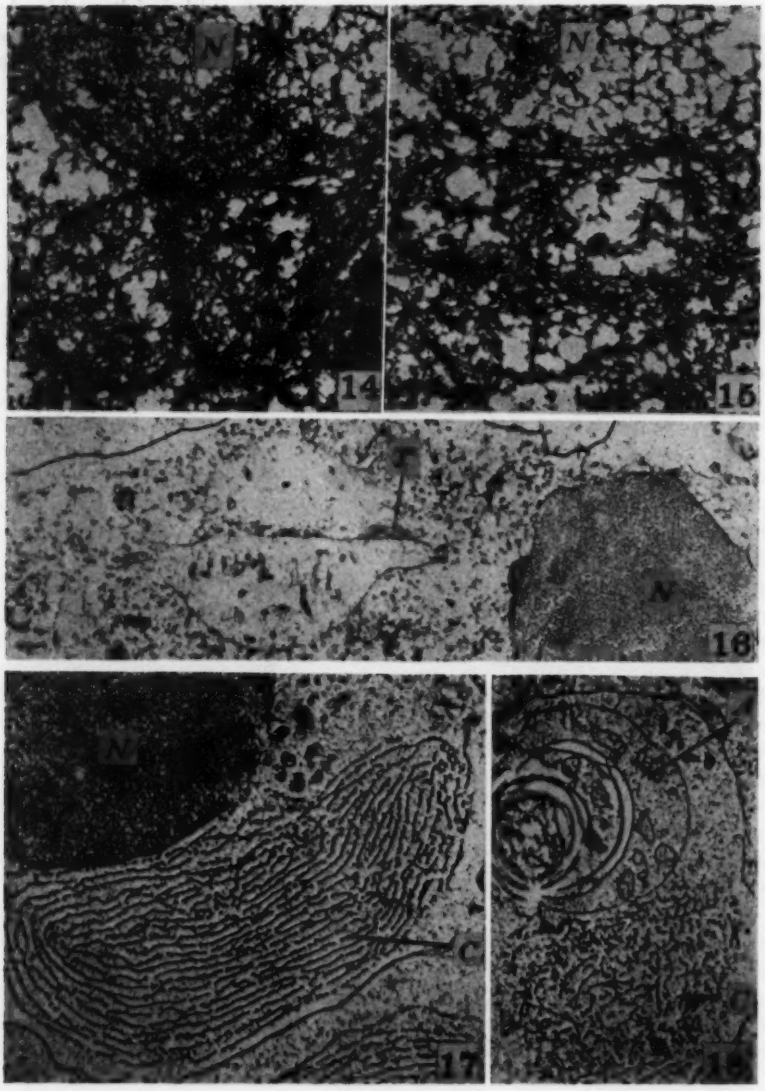


PLATE III

FIGURES 14 AND 15. Initial stages in sperm development, in which the nebenkern has lost its lamellae and divided, preparatory to moving into the middle piece. The nucleus is seen at N. Champy's fixation. Figure 14, × 10,000; Figure 15, × 5000.

the base of the nucleus, and a portion of the tail filament (T) is revealed between the halves of the elongating nebenkern.

Certain unidentified bodies were observed in the electron micrographs of the germ cells of the grasshopper (Figs. 17C and 18C). We believe these to be basophilic bodies which have been formed by a fusion of scattered basophilic material like that present in the cells of Figure 13C. These bodies show filaments or lamellae about 600 Å in width which are probably composed of double membranes. Between the filaments or lamellae are relatively structureless layers of matrix which measure on the average about 2170 Å in width. In Figure 18C the lamellae are seen in cross section. It is clear that this body is not a part of the nebenkern, since both bodies are sometimes revealed side by side in the same cell (Figs. 18A, C).

Discussion

Mitochondrial material is known to exist in many different forms such as granules, rods, filaments and vesicles; it is also known to be the locus of many important enzymatic functions. (For an excellent review of the morphology and biochemistry of mitochondria the reader is referred to the symposium on this subject appearing in the *J. of Histochem. and Cytochem.*, vol. 1, pp. 179 to 281, 1953.)

One of the most interesting types of mitochondrial bodies is the nebenkern which exists in the spermatids of certain insects (von la Valette St. George, 1886; Meves, 1900; Lewis and Robertson, 1916; Gatenby, 1917; Bowen, 1922; Payne, 1926; Pollister, 1930; Johnson, 1931 and others). The mitochondria in the young spermatid are composed of an outer chromophilic and an inner chromophobic part. They literally run together, giving rise to the nebenkern (Gatenby, 1917; Bowen, 1922) which, as the spermatid grows older, becomes differentiated into a series of ultramicroscopic lamellae that are separated by inter-lamellar spaces.

In preliminary observations on the living spermatid by means of the phase-contrast microscope, we were able to confirm much of the structure seen in the nebenkern of fixed and stained preparations. However, it is evident that the structure of the nebenkern seen in Figure 1B is difficult to correlate with that observed in the electron micrographs. Notwithstanding the fact that we have examined over 150 nebenkern in different stages of differentiation, we apparently have not seen the lamellar structure present in Figure 1B. That this is true is evidenced by the fact that the lamellae seen in electron micrographs measure only about 400 Å in width, a figure beyond the range of light microscope resolution. At present we are unable to explain this apparent discrepancy in structure of the nebenkern revealed by the light and electron microscopes; it may simply be that we have never observed under

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Figure 16. Stage in transformation of spermatid into spermatozoon. Note centriole at base of nucleus (N) and part of tail filament (T) between the elongating halves of the nebenkern. 2% osmic acid fixation. × 5000.

Figure 17. Basophilic body (C) cut through its long axis. Note electron-opaque lamellae or filaments and interlamellar spaces. The nucleus is shown at N. Buffered 2% osmic acid fixation. × 5000.

Figure 18. Body at C is of the same type as that in Figure 17C, but cut in cross section. The body at A is a developing nebenkern. Buffered 2% osmic acid fixation. × 6000.

the electron microscope a nebenkern possessing a structure of the type seen in Figure 1B.

The interesting part of this paper is, how does the ultramicroscopic structure of the nebenkern compare with that of other forms of mitochondrial material? It will be recalled that Palade (1952, 1953) demonstrated within the mitochondria a system of internal ridges or cristae that protrude perpendicularly from the inside surface of the membrane toward the interior of the mitochondria. Sjöstrand (1953) further observed these membranes to be double and to extend across the mitochondria. Since the cristae appear in the mitochondria of so many different types of cells, Palade (1953) suggests that they constitute a characteristic pattern of mitochondrial ultrastructure. However, in the mitochondria of the male germ cells of *Helix* instead of the characteristic cristae, a series of longitudinally oriented lamellae of about 400 Å in width were observed (Beams and Tahmisian, 1954). The same mitochondria show, when examined under polarized light, a positive birefringence in their longitudinal axis. If the grasshopper nebenkern is constructed like an onion (that is, the lamellae corresponding to the layers), then it may be said that the ultrastructure of both *Helix* germ cell mitochondria and grasshopper nebenkern are similar. However, it is difficult to determine what relationship, if any, exists between the lamellar structure of the nebenkern and the cristae structure of certain other mitochondria. Accordingly, it would seem that the exact type of internal ultramicroscopic structure in mitochondria material may vary, but that some form of structure must be present to provide compartments or increased intramitochondrial surface area which in some way enhances their enzymatic function. It is not known what role the mitochondrial material may play in the metabolism of the grasshopper spermatid. However, if there exists a correlation between structure and function within the mitochondria, as seems evident, the nebenkern with its remarkable structure should constitute a rewarding source of material for enzymatic studies.

It has long been known that certain material in the cytoplasm of many different types of cells has a special affinity for basic dyes. Hence, these bodies whose significance has not been wholly understood have been termed basophilic bodies or ergastoplasm (Wilson, 1925; Monné, 1948). Recently the electron microscope has revealed in different types of cells a group of filaments that are thought to be equivalent to the basophilic material (Dalton *et al.*, 1950; Bernhard *et al.*, 1952; Beams *et al.*, 1952, and others). According to Porter (1953), the fibrous nature of the basophilic material, which he has termed endoplasmic reticulum, is in reality an artifact, and the real structure is a system of canaliculi and vesiculated strands. The bodies that we have termed basophilic here seem to possess a structure similar to that in other types of cells. However, it is difficult to determine whether or not the lamellae are composed of single membranes or of double membranes with an intervening space between them. We are inclined to accept the latter view. Certainly the diffuse form of the basophilic material like that in Figure 13, appears vesicular.

It will be noted that in Champy-fixed material a more fibrous type of cytoplasm is revealed than occurs following fixation in buffered osmic acid; yet the structure and distribution of the lamellae within the nebenkern are much the same following either type of fixation.

Conclusions

1. The nebenkern of grasshopper spermatids arise by the fusion of mitochondria.

2. The mature nebenkern is composed of concentric lamellae about 400 Å in thickness. Some appear to be composed of double membranes. The lamellae are arranged like the layers in an onion and they alternate with layers of matrix.

3. As the spermatid transforms into a spermatozoon the nebenkern loses its lamellar structure, divides, and its two halves elongate down the middle piece of the developing sperm.

4. Some spermatids contain diffuse vesiculated bodies (endoplasmic reticulum) which are believed to be basophilic material. Other spermatids show large bodies composed of lamellae which are thought to represent a collection into one body of the basophilic material.

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STUDIES ON THE UREASE OF THE EGGS AND EMBRYOS OF THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS [1]

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From studies on the comparative physiology of animals, it has been found that the kind of nitrogen excretion products tend to be correlated with environmental conditions, the most important single factor being the availability of water. One might suppose, on the basis of environment, that if sea urchin eggs excrete nitrogen it would be excreted in the form of ammonia since ample water is available for the rapid removal of this substance from the eggs and developing embryos. There are, in fact, reports of ammonia excretion by sea urchin eggs. Örström (1941) demonstrated a marked but transitory excretion of ammonia by sea urchin eggs immediately after fertilization and provided some evidence that this is a result of the deamination of adenosine to inosine by adenosine deaminase. Hutchens *et al.* (1942) confirmed Örström's findings, but attributed the ammonia production to amino acid deamination. A number of workers have investigated nitrogen metabolism during later development with results which, though in some measure conflicting, suggest that the early development of the sea urchin egg is characterized by little if any nitrogen excretion. Hayes (1934) reported an increase in total nitrogen during the first four hours of the development of *Echinometra lucunter*, followed by a small decrease between the 6th and 24th hours of development. Ephrussi and Rapkine (1928) found a decrease in total nitrogen from 10.7% dry weight to 9.7% during the period of development between 0–40 hours of *Paracentrotus lividus*. Hayes' data are complicated by the possibility that variable amounts of the gelatinous coat of the eggs, a glycoprotein (Tyler, 1949a), may have been present in different stages, since no special account of this seems to have been taken in this work. Ephrussi and Rapkine's results, as indicated by Horowitz (1939), also show an increase of 5.6% in the dry weight of the embryos during the period of development mentioned which would virtually cancel out any nitrogen loss during this period. Horowitz (1939) presented data for the nitrogen content of the eggs of *Urechis caupo*, a marine echiuroid worm, indicating that the nitrogen content is constant up to 24 hours of development (feeding trochophore). Though this last mentioned work admittedly has no direct bearing on nitrogen excretion in the sea urchin egg, the environment of the two forms is similar and one might expect, therefore, a similarity in nitrogen excretion products. Finally, it is reported by Gustafson and Hasselberg (1951) that the nitrogen content of developing embryos of *Paracentrotus lividus* remains constant for the first 32 hours of development.

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The present investigation is concerned with the enzyme urease and metabolically related enzymes, and the possible relation between these enzymes and the low nitrogen excretion by the developing sea urchin embryo. Since urease is usually thought of as an "excretory" enzyme, its existence in an embryo which may not excrete nitrogen during the morphogenetic period leading to the larval stage becomes of some interest.

Materials and Methods

Eggs and sperm of *Strongylocentrotus purpuratus* were collected after the animals had been induced to spawn with isotonic KCl (Tyler, 1949b). The gelatinous coat of the eggs was removed with acid (pH 3.5) sea water (Tyler, 1949a), and the eggs were used if they were 90–100% fertilizable. Embryos were grown in approximately 1% suspensions at 11° C. in slowly rotating Erlenmeyer flasks. At the desired stage of development, samples were taken from the suspension. Swimming stages were concentrated by means of a specially constructed Lucite centrifuge similar to the Foerst plankton centrifuge. The embryos were frozen and stored for not more than 24 hours before *in vitro* assay for urease activity.

For *in vitro* determinations, the frozen material was homogenized and diluted with sea water to give a final concentration of about 0.5 ml. of packed eggs per 2.0 ml. of brei. Two milliliters of this were transferred to a 10-ml. screw-cap vial with 4.0 ml. of 0.5 *M* phosphate buffer at pH 7.4. At zero time, 1.0 ml. of a urea solution containing 100 mg. of urea was added. The reaction was allowed to proceed for one hour at 29.5° C., when the vials were chilled and centrifuged for 2–3 minutes at 3° C. Five milliliters of the supernatant were transferred to tubes containing 1 gm. of Permutit (after Folin). These were mixed for one minute, then washed three times with ammonia-free water. The Permutit was transferred to a Kirk-type micro-Kjehldahl still and alkalinized by the addition of 3 ml. of 20% NaOH. The ammonia liberated was distilled and titrated by the biiodate method of Ballentine and Gregg (1947). Kjehldahl nitrogen was determined on aliquots of the homogenates by the digestion method of Boell (1945) and the distillation and titration method of Ballentine and Gregg (1947).

For *in vivo* determinations, 6-ml. aliquots containing about 0.5 ml. of eggs were placed in a 10-ml. screw-cap vial, one ml. of sea water containing 100 mg. of urea was added and the eggs incubated for one hour at 18° C. The suspensions were then centrifuged lightly at 3° C. for one minute and 5.0-ml. aliquots of the supernatant were transferred to the Permutit. The eggs were washed with sea water, allowed to cytolyze in the original volume of cold distilled water and another series of 5.0-ml. aliquots taken from the supernatant. The cytolysis procedure permitted measuring ammonia that was produced by the reaction but failed to diffuse out of the eggs. The *in vivo* urease activity is taken as the sum of the ammonia liberated to the sea water plus that bound in the eggs and is expressed as micrograms of ammonia nitrogen per aliquot per hour.

Endogenous controls were run in all experiments and the data to be presented have been corrected by the subtraction of the control values. The endogenous ammonia production in breis was generally about 0.0075 microgram ammonia nitrogen per microgram egg nitrogen per hour, while for whole eggs, the usual figure was close to 17 micrograms ammonia nitrogen per aliquot per hour for unfertilized eggs

and 20 for fertilized, varying slightly with the number of eggs per aliquot present in different experiments.

Results

To ascertain the *in vitro* saturation point of the enzyme with substrate, two experiments with equal aliquots of eggs and varying urea concentrations were performed. The amount of urea per vial ranged from 2.5 to 20 mg. in one experiment, and from 10 to 90 mg. in the second experiment. The results indicate complete saturation with 30–50 mg. of urea but, as a safety factor, 100 mg. of urea per reaction vial were used in all *in vitro* determinations of urease activity.

Since urease in other organisms depends on reduced sulfhydryl groups for activity (Sumner and Poland, 1933), a check was made on the redox potential of the brei-buffer mixture using a Beckman pH meter equipped with a platinum electrode. The brei-buffer was found to have a redox potential of 150–170 mv. On the addition of one mg. of glutathione, this was lowered to 90–100 mv. The enzymatic activity was not appreciably changed in the presence of glutathione; values obtained were 0.0365 microgram ammonia nitrogen per microgram egg nitrogen per hour without glutathione and 0.0380 for the vial containing glutathione. As will be seen later, these results do not differ significantly from each other. In view of the failure of glutathione to increase the activity of the enzyme, and in view of the optimum redox potential of 100–200 mv. reported by Sizer and Tytell (1941) for jack bean urease, which is within the range obtained for the brei-buffer mixture, glutathione was not included in the subsequent assays.

To determine the reproducibility of the homogenizing, sampling and assay methods employed, embryos were raised to the hatching blastula stage and harvested. Three aliquots of these embryos were homogenized separately and their urease activities determined. Activities of 0.0361, 0.0368, and 0.0328 unit (microgram ammonia nitrogen/microgram egg nitrogen/hour) were obtained, from which it is concluded that the determinations are reliable to about 0.005 unit.

Using the conditions for assay established by the above experiments, determinations of the activity of the enzyme were made on breis of fertilized and unfertilized eggs and on various stages during the first 114 hours of development. These results are summarized in Figure 1. It is seen that while there is little change in the activity of breis between fertilized and unfertilized eggs, there is a drop of about 30% by the unhatched blastula stage, and that this level is maintained to the pluteus stage, at which time the animals are beginning to feed. Adult tissues were analyzed in order to see if any further change in the activity of the enzyme occurred. Ovary, from which the eggs had been removed by KCl-induced spawning, and gut were assayed. Activities of 0.0285 and 0.0585 unit, respectively, were found. It would seem that the activity of urease in the adult animal is maintained at approximately the level found during embryonic development.

That urease activity *in vitro* does not change when the eggs are fertilized is indicated above. There still exists the possibility of an *in vivo* change. To test this, equal aliquots of eggs were assayed in the manner described above. For two separate experiments, the results are: experiment 1, 126.8 micrograms ammonia nitrogen per hour per aliquot unfertilized and 99.9 fertilized; experiment 2, 127 unfertilized and 119 fertilized. These experiments show little or no change in *in vivo* urease

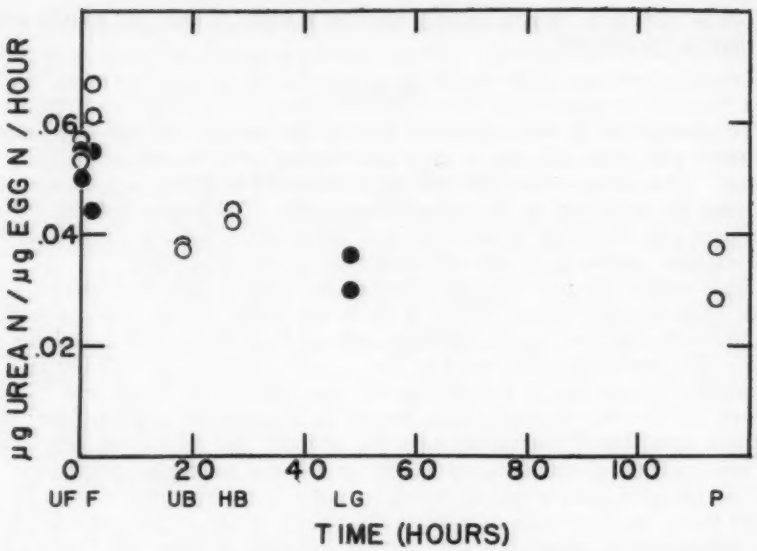

FIGURE 1. The *in vitro* activity of urease of *Strongylocentrotus purpuratus* as a function of time and developmental stage. UF, unfertilized; F, fertilized; HB, hatched blastula; LG, late gastrula; P, pluteus.

activity accompanying fertilization; the differences noted are certainly not comparable to the 4- to 6-fold increase found in respiratory rate on fertilization in sea urchins.

Experiments were done to determine if a change in urease activity resulted from homogenization. Equal aliquots of a suspension of unfertilized eggs were taken; one series was homogenized and the other was left intact. The temperature was 18° C. and the pH was 6.8 for the homogenized samples and 7.8 for the sea water in which the intact eggs were suspended. Though there is an apparent difference in the pH of the two samples, the enzyme may be considered to be acting at the same pH in both cases since the pH of the interior of the egg may be taken to be about 6.8 (Pandit and Chambers, 1932). The intact eggs produced 70 micrograms of ammonia nitrogen per hour per aliquot while the homogenized eggs produced 238.

There are numerous possible explanations for this four-fold increase in activity. One of these, the possibility that the eggs are relatively impermeable to urea, was tested by assaying for unfertilized *in vivo* activity with increasing amounts of urea. If urea is freely permeable, the *in vitro* saturation point (30 mg.) should also be reached *in vivo*. Two such experiments were done employing urea concentrations from 10 to 100 mg. per vial. The results, plotted in Figure 2, indicate that the enzyme is not saturated at 100 mg. of urea per vial (0.24 *M*) and therefore that the amount of substrate that reaches the enzyme is limited, presumably by permeability, assuming that urea is not being utilized by the eggs during the experiment. It should be noted that eggs incubated with urea were fertilizable to the same extent as endogenous controls (75–80%) after one or two washings with fresh sea water.

In order to have a more complete picture of the role of urease, it would be helpful to know how urea might be formed in the eggs. There are several pathways of urea formation, of which two are the ornithine cycle and the degradation of purines. Since the presence of a system such as the ornithine cycle, which would manufacture urea at the expense of energy only to have it immediately broken down again, seemed unlikely, it was decided to test for various enzymes of purine degradation. Homogenates of unfertilized eggs were examined qualitatively for the presence of adenase, xanthine oxidase, uricase, allantoinase, and allantoicase. Adenase was not demonstrable in more than trace amounts in two independent experiments using the method employed for urease, but with adenine as substrate. Xanthine oxidase was tested for using a modified Thunberg technique with both xanthine and hypoxanthine as substrates. Both substrates gave decolorization times of 10–15 minutes at 29° C. and pH 7.4, while in the absence of substrates, decolorization did not occur within 40 minutes. It is concluded that the enzyme is present. Uricase activity was measured in a Warburg apparatus. Two ml. of brei (0.5 ml. packed eggs per 2 ml.), to

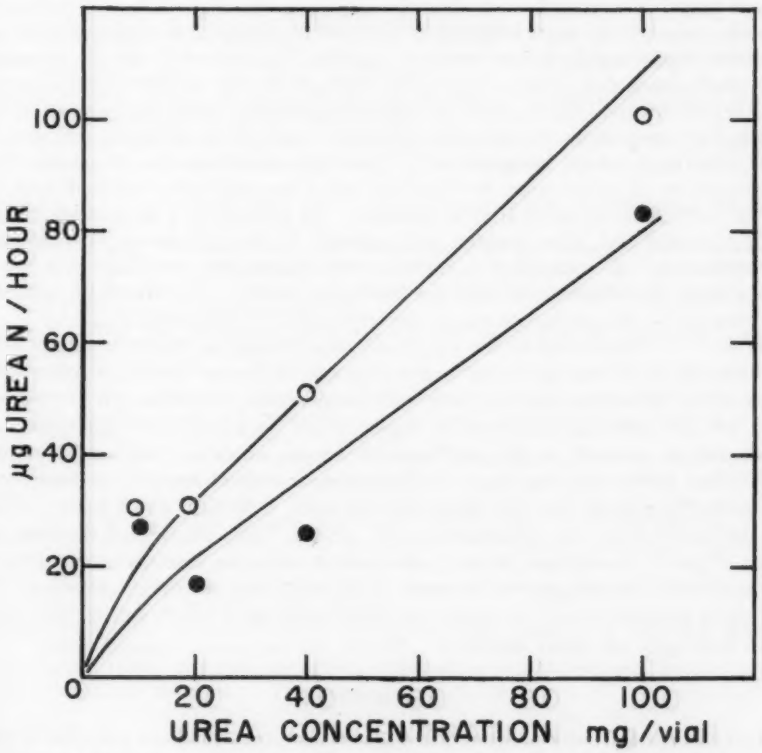

FIGURE 2. The *in vivo* activity of urease in unfertilized eggs of *Strongylocentrotus purpuratus* as a function of concentration of urea in the sea water.

which were added 20 mg. uric acid, gave an oxygen uptake at pH 9 and 30° C. of 10.0 and 14.0 microliters of O_2 per hour per flask in two separate experiments, whereas endogenous controls showed rates of 3.1 microliters of O_2 per hour per flask in both experiments; uricase is therefore present in the brei. Allantoinase and allantoicase were assayed simultaneously using the *in vitro* urease method, but with allantoin as substrate. Duplicate reaction vials contained 5.0 ml. McIlvaine's buffer at pH 7.4, 1.0 ml. undiluted egg homogenate, and 1.0 ml. allantoin solution containing 5 mg. of allantoin. In the duplicates 97.2 and 87.1 micrograms ammonia nitrogen were liberated in one hour at 22° C. These values have been corrected for a small endogenous ammonia production. It is concluded that allantoinase and allantoicase are present.

These experiments indicate that four of the enzymes metabolically related to urease are present in the unfertilized eggs. In addition, adenosine deaminase has been reported by Örström (1941) and Gustafson and Hasselberg (1951). It is probable, therefore, that the scheme of purine degradation typical of animal tissues exists in these eggs. With this conclusion, the function of urease in the sea urchin embryo might be expected to be that of completing the degradation of purines to ammonia, preparatory to its elimination from the organism as in a non-cleidoic system where water supply is not limiting. Against this, however, are the investigations cited earlier that indicate little or no nitrogen is lost by sea urchin embryos. The question of ammonia excretion in *Strongylocentrotus purpuratus* was examined directly by experiments in which the ammonia liberated by embryos into the sea water culturing medium was measured. One experiment covered the period from 20 minutes to 12 hours after fertilization, and a second was concerned with the interval from 24 to 60 hours after fertilization. In both, a 0.2% suspension of eggs was inseminated and, after washing, was cultured for the incubation period in fresh sea water at 11° C. and pH 8. Ammonia was determined on 5.0-ml. aliquots of the sea water both before and after the incubation period. No detectable ammonia was liberated by the larvae during the developmental periods tested.

With the establishment of the likelihood that nitrogen is excreted in very small quantities, if at all, during the early development of the sea urchin, it follows that urease is not primarily involved here with ammonotelic excretion. It is probable, then, that any ammonia produced by urease would be added to the metabolic pool of nitrogen to be used in the synthesis of various nitrogen compounds. Hultin (1950) has shown that the eggs of *Paracentrotus lividus* are able to incorporate N^{15}-labelled ammonia from sea water into proteins, a finding which tends to indicate the existence of such a metabolic pool. In this view, urease and the other enzymes of purine breakdown shown to be present are to be considered as factors in the mobilization of nitrogen for the many syntheses of the sea urchin embryos. An interesting possible source of purine for their action may be ribonucleic acid, with which these eggs are richly supplied.

Summary

1. The urease activity of sea urchin eggs and embryos has been followed *in vitro* to the pluteus stage. The activity was found to decline by about 30% upon reaching the blastula stage, after which it remains relatively constant.

2. Adult sea urchin tissues were found to contain urease of activity comparable to that of the eggs and embryos.

3. The *in vivo* activity of urease in fertilized and unfertilized eggs was compared, with little or no change on fertilization noted.

4. The *in vivo* activity was found to be considerably lower than *in vitro* activity when measured under the same conditions, and evidence indicates that the *in vivo* activity is limited, at least in part, by the rate of penetration of urea into the eggs.

5. Of the enzymes usually involved in the formation of urea from purines, xanthine oxidase, uricase, allantoinase and allantoicase are shown to be present in sea urchin eggs, whereas adenase was not demonstrable with the method employed.

6. No ammonia within the range detectable by the methods employed was liberated into sea water by embryos during the periods of 20 minutes to 12 hours after fertilization, and 24 hours to 60 hours after fertilization.

7. The results suggest that urease and the related enzymes function to add nitrogen from purines to the metabolic ammonia pool during the pre-feeding stages of development.

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CYTOLOGICAL AND CYTOCHEMICAL STUDIES OF OOGENESIS OF POPILIUS DISJUNCTUS ILLIGER (COLEOPTERA–POLYPHAGA)

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The cytological events encountered in studies of gametogenesis pose many problems of interest to the cytologist. Although studies of spermatogenesis and oogenesis are of equal importance from the biological viewpoint, apparently much less attention has been given to the latter since the early part of the present century.

As pointed out by Wilson (1928), among the more important aspects of oogenesis are such problems as the morphological and physiological relations of the developing oocyte to the so-called accessory cells associated with it, the mechanism of oocyte growth and the formation of the yolk substance. The present paper is concerned mainly with the early development of the oocyte and its relation to the accessory cells of the ovary. Studies on the phases of oocyte growth and yolk formation are in progress and the results will be published elsewhere.

Broadly speaking, insect ovaries may be classified into two main types depending upon the presence or absence of so-called nurse cells. Thus the panoistic type (nutritive cells are lacking) is characteristic of the Orthoptera, Odonata, Isoptera and Aphaniptera while the meroistic type is characteristic of Coleoptera, Neuroptera, Hymenoptera and Hemiptera.

If the location of the nurse cells is taken into consideration, then the meroistic type may further be subdivided into the polytrophic condition, where nutritive or nurse cells alternate with the oocytes, and the telotrophic condition, in which case the nurse cells are confined to the apices of the ovarioles.

There is, however, some confusion in the literature with respect to the correct placing of families, especially in the case of the Coleoptera. Thus Imms (1948), following the classification of Stein (1847), considers the sub-order Polyphaga to be characterized by the telotrophic condition and the polytrophic condition to be characteristic of the sub-order Adephaga. On the other hand, Wigglesworth (1939) reverses the classification of Imms and Stein and in this respect appears to be following Weber (1933). Finally, Deegener (1928) cites the Coleoptera-Polyphaga as possessing the telotrophic type of ovary.

Hence, according to Imms, *Popilius disjunctus* should possess ovaries of the telotrophic type, while according to Weber and Wigglesworth they should possess polytrophic ovaries. More recently, the structure of the ovariole was investigated by Krause (1946) who concluded that the ovarioles were of the typical telotrophic type. The cytological observations reported below support the conclusion that the ovarioles of *Popilius disjunctus* exhibit a modified telotrophic condition.

The relationship between nurse cells and oocytes has been investigated by many workers; see for instance the papers of Giardina, Korschelt, Nussbaum-Hilarowicz

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(reviewed in detail by Wilson, 1928), Shaffer (1920) and others. Such investigations suggested that nurse cells were abortive ova specialized for the formation of nutritive materials to supply the needs of the developing egg. This problem was re-investigated in the Hemiptera by Schrader and Leuchtenberger (1952) using cytochemical procedures. Their findings showed that the nurse cells did contribute material to the egg cytoplasm and furthermore that this nutritive substance was in part derived from the desoxyribose nucleic acid (DNA) of the nurse cell nuclei.

Materials and Methods

Specimens of *Popilius disjunctus* Illiger (Passalidae) were obtained from a biological supply house as required. In addition a number of *Macrodactylus subspinosus* Fabricius (Scarabaeidae) were collected in Newton, Massachusetts, and used to supplement the studies on *Popilius disjunctus* material. Both families are members of the superfamily Lamellicornia.

A. *Fixation*

The gonads were dissected free of tracheoles and fixed in Carnoy's solution (3:1) for two hours. The tissues were washed several times in absolute alcohol, cleared in benzene and embedded in 56–58° C. Tissuemat. Sections were cut at 10 μ and at 5 μ in order to provide sections containing whole nuclei and thin sections of nuclei for subjection to various cytochemical procedures. It should be pointed out that the dissections were performed immediately or within three days of receipt of each shipment.

B. *Staining*

1. *Nucleic acids*

a. The Feulgen reaction. The Feulgen reagent was prepared according to the method of Stowell (1945) and sections were stained for two hours at room temperature following optimal hydrolysis (12 minutes) in 1 N HCl at 60° C. All slides were stained in the same batch of reagent though at different times.

b. Methyl green staining. The dye was first purified by shaking a 2% aqueous solution with chloroform until the washings became colorless. A calculated aliquot of the purified dye solution was added to the phenol-glycerin mixture as used by Pollister and Leuchtenberger (1949) to give a final dye concentration of 0.2%. It has been shown by Pollister and Leuchtenberger (1949) and by Alfert (1952) that pretreatment of the slides with hot reagents causes a lowering of the amount of methyl green bound by nuclei. Therefore the staining reaction was carried out at 37° C. for 1½ hours. Slides were then rinsed in ice-cold water, blotted and rinsed in tertiary butyl alcohol and differentiated overnight in fresh tertiary butyl alcohol. This procedure was carried out in order to insure chemical staining (Michaelis, 1947).

c. Azure B. Sections were stained in 0.2% watery solution of azure B at pH 4.0 for 3 hours at 40° C., according to the method of Flax and Himes (1952). After rinsing, the slides were differentiated in the same manner as for methyl green.

Sections were also stained by the methyl green and azure procedures following treatment with ribonuclease (see below).

d. Toluidine Blue O. Preliminary investigations of basic staining were carried out by staining sections for 15 minutes in a 0.25% aqueous solution of toluidine blue followed by a short differentiation in absolute alcohol.

2. *Proteins*

a. Millon reaction. The reagents were prepared and used as described by Pollister (1950); see also Bryan (1951).

b. Fast green. A 0.1% solution of fast green was prepared and used as described by Schrader and Leuchtenberger (1950) and by Bryan (1951).

3. *Additional techniques*

a. Ribonuclease. Ribonuclease was obtained from the Worthington Biochemical Sales Company. The enzyme was boiled for five minutes in a few drops of saturated ammonium sulphate solution in order to remove any proteolytic impurities. The resulting solution was then diluted with redistilled water to give a final enzyme concentration of 0.2 mgm. per ml. Slides were exposed to the enzyme solution for four hours at 45° C. Control slides were immersed in ammonium sulphate solution (0.2 mgm. per ml.) but at the same temperature and for the same amount of time as the test slides.

b. Periodic acid-Schiff reaction (P.A.S.). The reagents were prepared according to McManus (1948). Sections were oxidized with periodic acid (0.5%) for 5 minutes, rinsed in distilled water and stained in leucobasic fuchsin solution for 15 minutes. After staining in the leucobasic fuchsin solution the slides were passed through a bleach bath (3 changes of 10 minutes each) consisting of 10 ml. of 1 N HCl, 10 ml. of a 10% $K_2S_2O_5$ solution and 180 ml. of distilled water.

Following this treatment the slides were washed in water, dehydrated and mounted. In certain cases methyl green was used as a counterstain.

4. *Photometry*

Measurements of the amount of dye bound or the color produced in the tissue sections were made with an apparatus essentially the same as that described by Pollister and Ris (1947) and by Bryan (1951). However, instead of a Weston micro-ammeter, a General Electric micro-ammeter having a sensitivity of 0.0014 micro-ampere per millimeter was used to measure the photomultiplier output. By means of a calibrated Ayrton shunt (total resistance 10,000 ohms) the sensitivity level of the meter was adjusted to the optimal range for recording the output of the photomultiplier tube. Interference filters were used to isolate from the spectrum of an AH_4 mercury lamp the spectral lines used in making photometric measurements. Each line isolated was close to the absorption maximum of the color reaction concerned.

It was necessary to compare data obtained from slides prepared at different times. Therefore, in order to check on the reproducibility of the reactions used, sections from one ovary were mounted on each slide and used as a standard. The extinction value of the blank prepared for the Feulgen reaction was found to be negligible.

5. *Computation*

The transmittance data were obtained by the "plug" method described by Ris and Mirsky (1949) and by Swift (1950). In addition, whenever warranted, the

mean optical path (MOP) of each nucleus was determined and used in the computations as described by Bryan (1951). The transmittance data were converted into extinction values ($\log_{10}$ 1/T) and the extinction data were computed as described by Bryan (1951).

It is self-evident that the amounts of substance computed from extinction data are expressed in arbitrary units which are, however, related to the absolute amounts of absorbing substance present.

Results

A. *Cytological Studies*

Since there is in the literature some confusion with respect to the classification of the Coleoptera, based on the structure of the ovariole, the cytological findings are reported in some detail.

In *Popilius disjunctus* the typical number of ovarioles is two per ovary while in *Macrodactylus subspinosus* it is six. The histological structure is essentially similar in both species. Unless otherwise indicated, the following observations are based on studies of *Popilius* material.

Histologically speaking, each ovariole may be divided into two major regions—the germarium and the vitellarium. A longitudinal section of an ovariole is depicted in Figure 1.

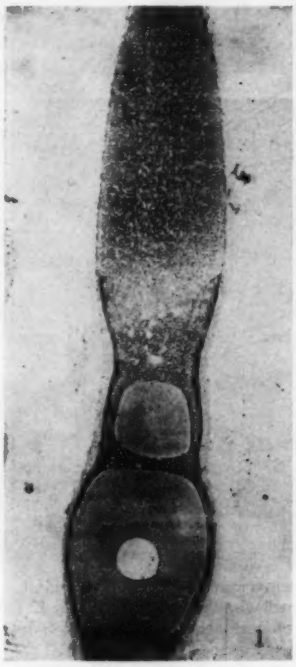


Figure 1. Longitudinal section through an ovariole from a mature adult. Stained with azure B. Photo 100 ×.

The germarium

At the apex of each ovariole the germarium becomes drawn out into an attenuated filament in the form of a spiral. As may be seen from Figure 2, this region in the immature female presents a distinctly different appearance from the

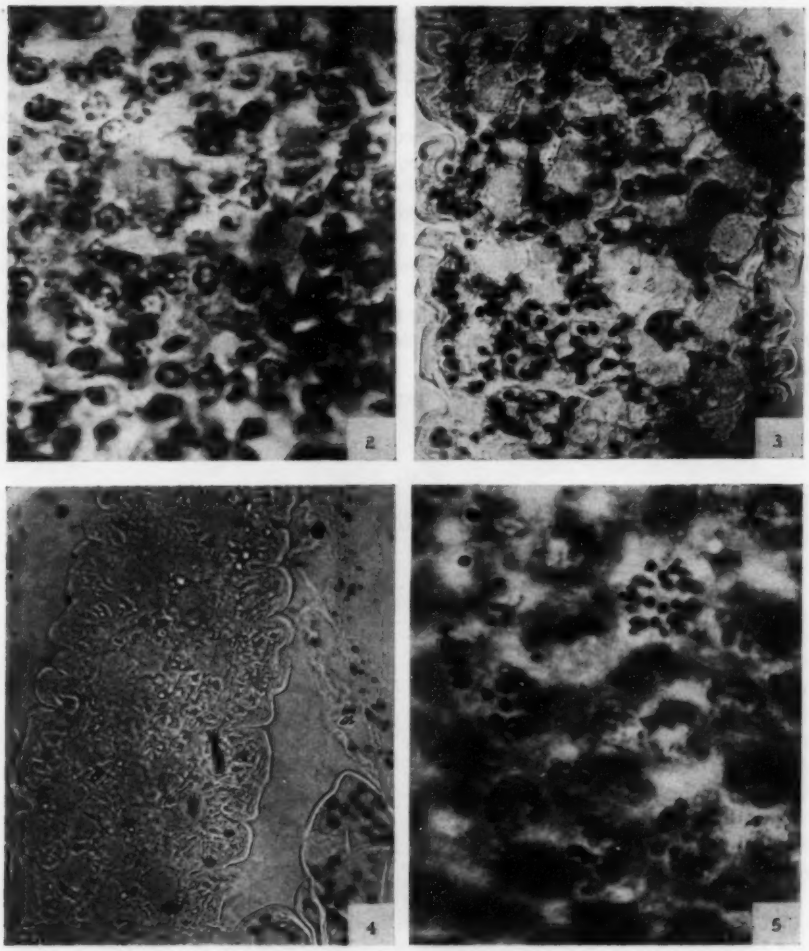


FIGURE 2. Spiral tip of germarium of an immature adult. Note the masses of acidophilic material. Feulgen counter-stained with fast green. Photo 1200 ×.

FIGURE 3. Similar region of germarium but from an individual a few weeks older than in the case of Figure 2. Stained with azure B. Photo 600 ×.

FIGURE 4. Spiral tip of germarium from a breeding female. Feulgen stain. Photo 400 ×.

FIGURE 5. Oogonial mitoses in germarium of immature adult. Feulgen counter-stained with fast green. Photo 3000 ×.

rest of the germarium. The cells appear to surround large masses of material which stains strongly with acid dyes as well as giving a positive Schiff (P.A.S.) reaction.

In older individuals the nuclei are slightly smaller but the chromatin is much more homogeneous. This condition is depicted in Figure 3. The apical region

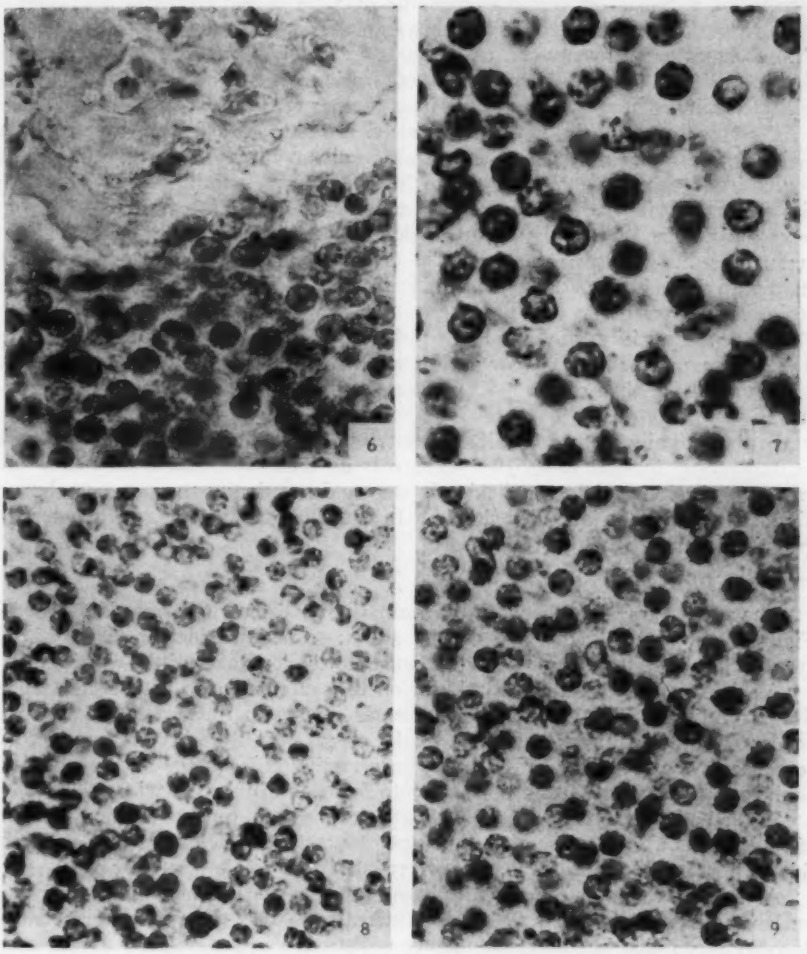


FIGURE 6. The junction of the spiral tip with the body of the germarium. Feulgen. 1200 ×.

FIGURE 7. Nuclei from the body of the germarium of a young adult, showing partially condensed chromosomes. Feulgen stain. Photo 1700 ×.

FIGURE 8. Distal portion of germarium of a young adult. Feulgen stain. Photo 1000 ×.

FIGURE 9. Proximal portion of germarium from same ovariole as in the preceding figure. Feulgen stain. Photo 1000 ×.

of an ovariole taken from a laying female is illustrated in Figure 4. It is readily seen that the apical region is now almost devoid of nuclei. There is no evidence that this condition is brought about by pycnotic degeneration of the nuclei.

In the body of the germarium the cells are small and present a closely packed appearance; the nuclei are spherical and there is but little cytoplasm. Mitotic figures are abundant in this region of the ovariole of sexually immature females (Fig. 5). Counts of oogonial metaphase plates indicate the diploid number of chromosomes to be twenty-six, as previously reported by Shaffer (1917) and by Smith (1953). On the other hand, examination of ovarioles of mature females indicated mitotic figures to be absent from the germarium.

Close to the spiral tip, most of the nuclei exhibit a typical interphase condition (Fig. 6); however, nucleoli are extremely small or absent. More posteriorly, in young females, most nuclei appear to have been arrested in late prophase, the chromosomes being partially condensed and peripherally located (Fig. 7). Here, in sections stained by the Feulgen technique followed by counterstaining with fast green, there may be seen small globules or, occasionally, thread-like bodies which stain with the acid dye. This acidophilic material is usually located in the central region of the nucleus.

While the nuclei at any one level in the germarium show a uniformity of size, those in the proximal part show an increase in size such that the average nuclear volume is twice that of the younger (more distal) nuclei—compare Figures 8 and 9.

In the sexually mature female, the oocytes adnate to the vitellarium appear to be undergoing meiosis (Fig. 10). There is no indication that development progresses beyond the diplotene-diakinesis stage. Instead, the chromosomes become diffuse and cannot be identified clearly. It is at this time that the oocyte enters the growth phase.

The vitellarium

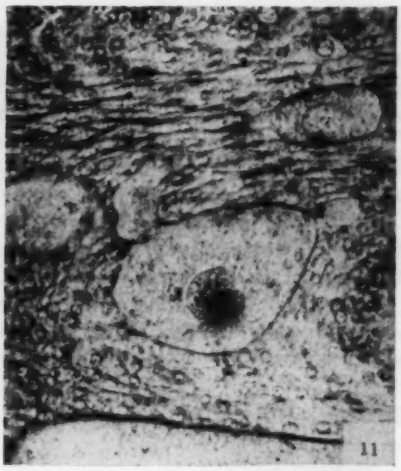
The zone of transition between the germarium and the vitellarium is depicted in Figure 11. Here the enlarging oocytes appear to be embedded in layers of cells greatly elongated in a direction perpendicular to the long axis of the ovariole.

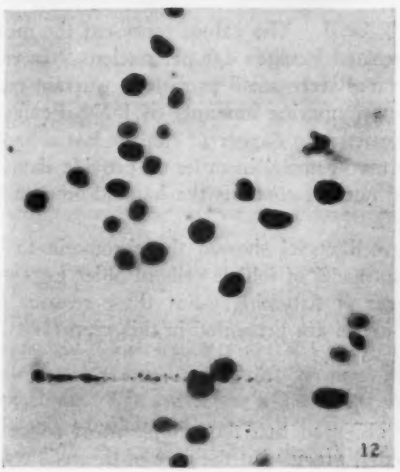
As the oocytes pass through this region they often develop cytoplasmic finger-like projections (Fig. 11). These processes cannot be traced beyond the distal border of the transitional zone. It would appear that they may be produced in response to the squeezing effect of the surrounding cells on the oocyte cytoplasm as the egg cells move through this zone into the body of the vitellarium.

In the body of the vitellarium each oocyte can be seen to have acquired a sheath of follicle cells. In the case of the younger oocytes, the follicle is several cell layers thick whilst in older oocytes it has been reduced to a single layer of cells.

As the oocyte enters the growth phase there is a marked increase in volume of nucleus and cytoplasm. As the nucleus increases in size, there is a corresponding decrease in staining intensity; the mature nucleus is Feulgen-negative and does not stain with basic dyes. There is no evidence of marked nucleolar activity during the oocyte growth phase. If, however, protein stains are used, then it can be seen that the nucleus stains darkly at all times. As can be seen from Figure 11 the nucleus at first has a granular appearance. In older eggs, the protein stain is distributed quite homogeneously throughout the nucleus.

Cytological examination of the germarium (including the spiral tip) failed to reveal any signs of extensive cellular degeneration. No indications were found of the existence of plasmatic strands connecting the germarium with the developing eggs. In this respect the ovaries of *Popilius* differ markedly from the condition





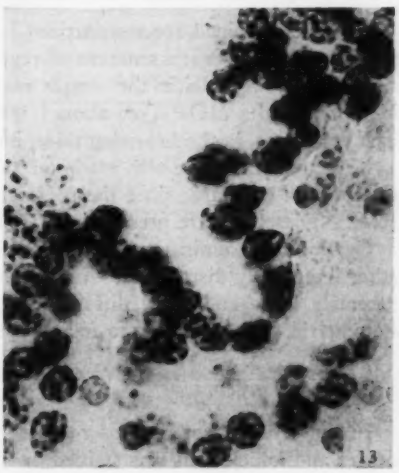


FIGURE 10. Portion of germarium adnate to the vitellarium, showing meiotic figures. Feulgen counter-stained with fast green. Photo 1500 ×.

FIGURE 11. The zone of transition between the germarium and vitellarium, showing an oocyte with cytoplasmic processes. Stained with the bromphenol blue-mercury reagent. Photo 500 ×.

FIGURE 12. Epithelium of the lateral oviduct, showing polyploid nuclei; compare with Figure 13. Feulgen stain. Photo 1200 ×.

FIGURE 13. Oviduct epithelium from the same section as figure 12. Note the clumps of heterochromatin. Feulgen stain. Photo 1200 ×.

found in the Hemiptera. Subsequent examination of the *Macrodactylus* material agrees with the observation made on the *Popilius* material.

In the vitellarium the eggs are arranged in a single row. Posterior to the oldest egg the ovariole is closed by a plug of epithelial cells which is ruptured at the commencement of oviposition.

Eggs from each pair of ovarioles are released into the lateral oviducts which unite to form the duct leading to the exterior. The nuclei of the cells lining these ducts exhibit several interesting phenomena. Here nuclear volumes fall into several classes simulating a polyploid series. While the smaller nuclei tend to be homogeneous (Fig. 12), the larger nuclei have a markedly different appearance (Fig. 13). Many strands of chromatin may be seen and, in most cases, they can be traced to clumps of heterochromatin located close to the inner surface of the nuclear membrane. Counts of these clumps, while somewhat variable, correspond closely to the diploid number of chromosomes. The amount of chromatin in each nucleus would appear to be several times as great as that found in the nuclei of other tissues.

It is obvious from the foregoing observations that the structure of the *Popilius* ovariole is quite different from that encountered in the Hemiptera and in other insects possessing the telotrophic type of ovariole.

B. *Cytochemical findings*

1. *DNA-Feulgen*

The data obtained are summarized in Table I. The values represent the mean extinctions, volumes and amounts of regenerated Feulgen dye per nucleus. In certain cases a few nuclei in the sample measured were small enough to warrant consideration of the MOP (see above) when computing amounts of DNA-Feulgen. Such values are, in the following table, indicated by a dagger (†) in the last column.

Measurements were also made on spermatid nuclei in order to provide data to be used as a standard, since the spermatid nucleus contains the haploid amount of DNA. These data are presented in Table I.

Cytological examination of the follicle cell nuclei showed the chromatin to be rather irregularly distributed. In addition, nuclei of follicle cells of older eggs are extremely large and show different degrees of flattening. For these reasons no photometric data pertaining to follicle cell nuclei are presented in this report.

2. *Methyl green*

In the present work it was repeatedly observed that the cytoplasm of oogonia, oocytes and follicle cells was stained by methyl green. In the case of the cells comprising the body of the germarium strong staining of the cytoplasm was observed; whereas the developing oocytes in the vitellarium, together with the investing follicle cells, evinced much weaker reactions. Overnight pretreatment of slides in the cold with dilute perchloric acid (Ogur and Rosen, 1950) abolished cytoplasmic staining. Complete suppression of cytoplasmic staining was achieved also by the use of the enzyme ribonuclease (see above).

TABLE I

DNA-Feulgen content of nuclei

Tissue and stage	Class	No. of nuclei measured	Mean extinction E_{440}	Mean nuclear volume microns³	Mean amounts in arbitrary units
Testis-spermatid	I (n)	21	0.041	40.8	0.53±0.03*
Ovary: spiral tip of germarium					
(a) Immature adult	II (2n)	8	0.096	54	1.09±0.09
II→III	II *a*	24	0.132	60.5	1.60±0.02
	III (4n)	10	0.170	58.6	2.14±0.08
(b) Mature adult	II (2n)	16	0.190	43.1	1.12±0.03*
II→III	II *a*	13	0.143	44	1.46±0.03*
	III (4n)	2	0.162	43.1	2.05
Body of germarium					
(a) Immature adult Oogonia	II (2n)	19	0.086	70.4	1.11±0.04
II→III	II *a*	20	0.146	58.4	1.73±0.02
	III (4n)	11	0.191	64.6	2.42±0.08
(b) Mature adult oocytes—distal portion of germarium	III (4n)	20	0.188	58.4	2.27±0.09
III→IV	III *a*	2	0.232	68.5	3.13
	IV (8n)	3	0.350	52.8	4.96
Oocytes—proximal portion of germarium	II (2n)	3	0.090	52.8	1.35
III→IV	III (4n)	23	0.126	106	2.10±0.09
	III *a*	1	0.144	124	3.09
Epithelium of lateral oviduct	II (2n)	3	0.226	17.5	1.23
	III (4n)	15	0.269	30.4	2.06±0.13
	IV (8n)	27	0.461	39.9	4.31±0.14*
	V (16n)	29	0.641	72.5	8.86±0.18

* MOP considered.

3. *Azure B*

When used as described by Flax and Himes (1952), azure B stains DNA orthochromatically blue-green and RNA metachromatically purple. As is the case with other basic dyes, azure B failed to stain the nuclei of oocytes contained within the vitellarium. The pattern of cytoplasmic staining, obtained with azure B, is essentially the same as that obtained with methyl green. The staining of the cytoplasm was completely abolished by a prior digestion of the slides with ribonuclease.

4. *Protein methods*

It was found that the masses of acellular material in the spiral tip of the germarium stained quite strongly with fast green and less intensely with the Millon reagent.

In very young oocytes, before the growth phase has commenced, the threads of chromatin stain quite strongly while the cytoplasm is only weakly stained. In later

oocytes, the nucleus presents a more homogeneous picture and, in contrast to the results obtained with basic dyes, still stains in an intense manner. In such nuclei the individual chromosomes can no longer be identified. As stated earlier, the nucleolar system does not show signs of intense or prolonged activity; only in a few older egg cells have small intranuclear bodies been observed. Studies on the protein content of oocytes are still going on and the results will be published at a later date.

5. *The periodic acid-Schiff reaction*

It was found that in the spiral tip of the germarium the acellular material which stained strongly with fast green also gave a strongly positive reaction with the Schiff reagent. In addition, small amounts of Schiff-positive material were distributed throughout the body of the germarium in an irregular fashion. In the cytoplasm of intermediate oocytes may be seen Schiff-positive droplets and granules. These structures are peripherally located, lying just below the surface of the egg. They are, therefore, in close proximity to the inner surface of the surrounding follicle cells. In much older eggs, where the chorion is present, the cytoplasmic Schiff-positive material is more uniformly distributed. It is also of interest that the tunica propria and the chorion give a strongly positive Schiff reaction.

Discussion

1. *DNA-Feulgen*

The germarium. From the data summarized in Table I it may be inferred that, in the immature female, the body of the germarium is essentially filled with oogonial cells multiplying rapidly by mitosis. The data indicate that the oogonial nuclei build up their DNA content to four times the haploid value prior to division—at which time the amount falls to that of the diploid value. Similar findings, in various animal tissues, have previously been reported by Swift (1950) and others.

The apical region of the germarium presents a similar picture of mitotic activity. Often strands of cells from the apical region appear to interdigitate with the most anterior layers of oogonial cells contained within the body of the germarium. The close correspondence of the photometric data, together with the cytological observations, suggests that the type of nucleus found in each region may be, in fact, identical.

In the case of the mature female the spiral tip, though mitotic figures were absent, still contains nuclei which possess an amount of DNA in excess of the diploid value. As reference to Table I shows, only two nuclei were found with the Class III amount of DNA; whereas Class II *a* (intermediate) and Class II nuclei were found in approximately equal numbers. The data show that mitotic activity continues in this region longer than in the body of the germarium. Perhaps the lack of Class III nuclei is an indication that mitosis has, by this time, slowed down or ceased.

According to Krause (1947), early in development a small group of primordial germ cells forms a knob-like structure at the distal tip of each arm of the Y-shaped gonad anlage. Later this knob gives rise to the spiral tip of the germarium. The central and basal portions of each anlage become hollowed out to form the cylindrical egg tube. This result is achieved by extensive cellular degeneration. Such degenerative changes were not reported to occur in the future spiral tip of the germarium.

Hence it may well be that most, if not all, of the functional primordial germ cells are separated from the rest of the anlage very early in development. Then it would follow that their derivatives, the definitive oogonia, as they are formed, pass into the body of the germarium where they multiply still further.

It is also possible that the primordial sex cells contained within the knob-like tip of the anlage are destined to become trophocytes or nurse cells. Such an interpretation would not be necessarily in conflict with the photometric data. It would be expected that such cells should undergo mitosis, during the time that oogonial divisions were taking place, in order to supply adequately the later nutritional needs of the oocytes.

On purely structural grounds, however, it would appear that such an interpretation should not be given undue emphasis for it is obvious that any nutritive material, in order to reach the growing egg cells, must diffuse through the entire body of the germarium.

At the present time it is not possible to discount fully the latter alternative. Though the presence of large amounts of PAS-positive substance in the apex of the germarium is suggestive of a trophic function for this region, it is clear that the apical zone cannot play as important a role in this respect, as does the end zone in the Hemiptera (see Schrader and Leuchtenberger, 1952). Such a conclusion is further supported by the absence of plasmatic strands connecting the apical region with the developing oocytes.

With respect to the DNA content, the condition prevailing in the body of the germarium of mature females is quite different. The data indicate that, with few exceptions, all the nuclei measured now have the Class III amount of DNA.

In the case of the proximal region three nuclei, out of a sample of twenty-seven, were found to possess the diploid amount of DNA. It is possible that these nuclei were the products of recent mitotic divisions and that the DNA content had not yet been built up to the final amount. It is also possible that these nuclei may be early follicle cell nuclei which were measured in error although this does not seem very probable for cytological reasons.

It was also observed that occasionally nuclei were encountered which were stained much more darkly than the typical oocyte nucleus, but which were of about the same size. In these observations perhaps lies the explanation for the finding, in the germarium, of nuclei possessing more than the tetraploid amount of DNA. It would appear likely that such nuclei, present in small numbers, are abnormal and do not give rise to mature eggs.

The vitellarium. The chromatin of the nuclei of oocytes in the zone of transition is distributed in an irregular manner. For this reason it was not possible to obtain valid photometric data.

It is well known that as the oocyte nucleus increases in volume, the intensity of the Feulgen stain and the degree of basophilia diminish. Nuclei of the developing oocytes in the vitellarium do not stain with basic dyes and also appear to be Feulgen-negative. Results such as these have often been interpreted to mean that there is no DNA in the nuclei of oocytes during the phase of growth. It is more probable that the decrease in intensity of the Feulgen reaction is attributable to a "diluting effect" of the increasing amounts of protein in the egg cell nucleus. This problem has been considered in more detail by Alfert (1950), and further remarks need not be made here.

As pointed out earlier, the follicle cell nuclei are not well suited to photometric needs. It is true that in the case of the younger follicles the nuclei are smaller and the chromatin is fairly homogeneously distributed. However, in these cases it was found that there was considerable overlapping of nuclei in the sections prepared. The degree of overlapping was such as to preclude the carrying out of photometric studies. It is of interest that, unlike the conditions obtaining in other insects, the cells of the more mature follicles do not appear to be multinucleate. Instead the volume of each nucleus has undergone a marked increase for it was found that the volumes of these nuclei were of the order of 1000 μ^3.

The lateral oviducts. Photometric measurements of DNA-Feulgen were made on nuclei of the epithelial lining of the lateral oviducts. The data are presented, in summary form, in Table I. It should be pointed out that only nuclei in which the chromatin was homogeneously distributed were chosen for measurement; no attempts were made to obtain data from nuclei such as those depicted in Figure 13.

It may be seen that the epithelial cell nuclei appear to contain amounts of DNA in multiples of the Class II (diploid) amount. It is also evident that nuclei containing the Class II amount are very infrequently met with, while nuclei possessing the Class IV or Class V amount of DNA constitute the most frequent classes.

The data suggest that there is a tendency for an increase in DNA content to be accompanied by an increase in nuclear volume. Whether or not a similar change in protein content occurs is unknown at the present time. Examination of the tissue involved failed to reveal any indication of metaphase plates or other phases of division. It is, therefore, quite probable that the mechanism underlying the formation of such nuclei, possessing amounts of DNA in multiples of the diploid amount, may be one of endomitosis. That the epithelial cell nuclei are endopolyploid in nature is further supported by the observations presented in the cytological section of this report.

This occurrence of polyploid cells in insect tissues is not unique for, as is well known, endopolyploid nuclei have been encountered in a variety of insects. Briefly, they have been reported by Geitler (1937) to occur in various tissues of *Gerris lateralis* and similar findings have been described by Berger (1938) with respect to the larval intestine of *Culex pipiens*. More recently, White (1951) has shown endopolyploid nuclei to be present in the testicular envelope of certain grasshoppers.

It is not possible at the present time to attach any functional significance to the present findings. Examination of the cytoplasm of these cells precluded the idea that the epithelial tissue may possess a glandular function. The existence of polyploid nuclei in the lateral oviduct tissue is perhaps a cytological indication of ageing.

2. *General considerations*

The basic dyes, azure B and toluidine blue, were used in conjunction with ribonuclease to study the cytoplasmic basophilia of oocytes and oogonia. During the course of these studies it was noted that the acellular material in the apex of the germarium stained, at best, very faintly with the dyes used. These observations indicate that the acellular material does not contain large amounts of RNA.

It would appear to be of some significance that the cytoplasmic basophilia of the follicle was found to be particularly intense during the periods of oocyte growth and chorion formation. It follows, therefore, that the follicle may be instrumental in

supplying the growing egg with nutritive material. It would also appear that follicle cells are intimately concerned with the formation of the chorion.

It is a striking fact that in *Popilius,* nucleoli are never very prominent components of the nucleus. Perhaps the variable form of the nucleoli may be attributed to the absence of "effective" nucleolar organizing regions. Such a conclusion would not be in conflict with the observations since Heitz (1931) has reported the formation of nucleoli despite the fact that nucleolar regions were lacking.

That the nucleolar system, as envisaged by the Caspersson school, does not achieve prominence during the period in which oocytes are rapidly increasing in size may argue against the idea that, in *Popilius,* nucleoli play an important role in the growth process. At the present time, however, the mechanism of oocyte growth must remain an open question.

The life history of *Popilius* has been studied by Riley (1872), Wheeler (1923), Gray (1946) and others. Oviposition commences during May and continues until early July, the peak of activity being in June. According to Gray (1946), relatively few eggs are laid by any one female. The average number appears to be of the order of thirty-five and sixty appears to be the maximum.

From mid-August until late in October immature red-colored adults are found in the colonies; later than this, all individuals appear to be the black-colored mature adults. It is of interest that in the present work, no beetles taken from the colonies later than August proved to be the mature adults of the previous spring, indicating that death must occur shortly after the end of the reproductive phase.

Examination of the ovarioles from immature adults taken in late September revealed the presence of developing eggs in the vitellarium. Ovarioles of immature individuals taken a few weeks earlier proved to contain numerous oocytes undergoing meiosis. The stages found ranged from zygotene to diakinesis. It should be stated that the fixative used (Carnoy 3:1) does not preserve the meiotic figures well enough to permit identification of the stages with absolute certainty.

From the foregoing observations it would appear that the first eggs laid must have been present in the vitellarium for some months prior to the breeding season. However, this does not seem to be the case, for it was repeatedly observed that in individuals taken over the winter months, the oldest eggs appeared to be undergoing degeneration and resorption. In one case, the posterior part of the vitellarium had been invaded by phagocytes, suggesting that phagocytosis may be an important factor in the resorptive process. It would appear then that during the winter months mature females may actually rely upon the almost mature eggs and their follicles as a source of supplementary nutritive material. Individuals obtained a few weeks prior to the commencement of the breeding season failed to reveal any indication of degenerative processes in the vitellarium.

That unfavorable environmental conditions may be responsible for the degenerative process is perhaps further suggested by the following observations. Ovarioles taken from *breeding* females immediately upon receipt appear to be quite normal whereas indications of oocyte degeneration are apparent if such females are kept under laboratory conditions for longer than ten days. In addition, there are the observations of Gray (1946) to the effect that under laboratory conditions females do not lay for more than six or seven days and, furthermore, the last eggs to be laid under such conditions are usually abnormal in appearance.

SUMMARY

1. The cytology of the ovarioles of *Popilius*, together with their associated ducts, is described.

2. Cytological and cytochemical studies of the germarium suggest that the ovariole constitutes a modified telotrophic type.

3. Microspectrophotometric measurements of nuclei, stained by the Feulgen reaction, indicate that during the interphase period oogonial nuclei build up their DNA content to twice the diploid amount and that after division the daughter nuclei each contain the diploid amount. The data also indicate that in mature females primary oocyte nuclei contain the tetraploid amount of DNA.

4. Cytological observations, together with photometric measurements, suggest that the epithelium of the lateral oviducts is an endopolyploid tissue.

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WATER FILTRATION BY THE BAY SCALLOP, PECTEN IRRADIANS, AS OBSERVED WITH THE USE OF RADIOACTIVE PLANKTON [1]

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Studies of the movement of sea water through the mantle cavity by lamellibranch molluscs and the efficiency of the gills in removing particulate matter from this flow are of considerable interest to biologists concerned with investigations of the feeding activities of these bivalves. Only through a more complete understanding of the factors controlling the rate of water propulsion, the retention of particles by the gills, and the ingestion of filtered material can we approach problems in the nutritional physiology of these animals.

The rate of water propulsion of a number of species of lamellibranchs has been reported by investigators using either direct measurement techniques or indirect methods based on the reduction of the number of particles in a suspension in which the animals have been placed. Considerable work has been done on the factors affecting the rate of pumping of oysters using direct measurements of the rate of flow following, in general, the experimental arrangement described by Galtsoff, Chipman, Hasler and Engle (1938). The important part of this technique is the use of an "apron" developed by Nelson (1936). In many lamellibranchs direct measurements cannot be made and indirect methods have been widely used. About the first of these measurements that may be considered as truly quantitative was that of Fox, Sverdrup and Cunningham (1937). Their experiments were based on the chemical determination of changes in the calcium content of suspensions of calcium carbonate in which *Mytilus californianus* were placed. The use of plankton in suspension with photoelectric determination of changes in concentration resulting from the activity of the animals was suggested by Jørgensen (1943). Colloidal graphite was used by him in later studies (1949a, 1949b, 1952, 1953) in measurement of conditions affecting the pumping rate of filter-feeding invertebrates and the relation of this rate to metabolic activity. Rao (1953) used these techniques to advantage in his studies on the rate of water filtration of *Mytilus californianus* from different latitudes.

With the availability of methods of labeling plankton cells with radioactive isotopes and the extremely accurate technique of measuring changes in cell numbers based on the detection of small amounts of radioactivity contained in such cells, it seemed advantageous to employ these methods in studies of the filtration rates of filter-feeding invertebrates. Consequently, we have used plankton containing radioactive phosphorus in studies of the rate of water propulsion of the bay scallop, *Pecten irradians* Lamarck, one of the more active species of lamellibranchs, which could lead

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to further work on the factors concerned with filtration rate and feeding in these and other related marine molluscs.

Materials and Methods

The scallops were collected from the vicinity of the Shellfish Laboratory of the U. S. Fish and Wildlife Service at Beaufort, North Carolina. They varied in size according to their age. They were all of the same year class, spawn of the fall of 1952, and consequently were rather uniform in size for any date of collecting. No very small scallops nor very large scallops were used. The smallest were those studied in June of 1953, and the largest those collected in October, 1953. Spawning of some scallops took place in the laboratory jars starting August 7 and was observed as late as early October. The biology of this species is well covered in a paper by Gutsell (1930).

Observations were made on single scallops immersed in suspensions of plankton cells in sea water. The volume of the suspension was usually 3 liters. With the small scallops a few observations were made in volumes of 1.5 liters, while the largest were tested in 6-liter volumes. The suspensions were well stirred by a stream of air bubbles. It was necessary to fasten the scallops to an L-shaped glass rod to prevent their swimming about. Since the scallop is very sensitive to the movement of an object in the near vicinity, the jars were well enclosed in a wrapping. When not under observation, the scallops were kept in running sea water.

The salinity of the sea water in the laboratory ranged from 31 to 36 parts per thousand, usually being about 34 parts per thousand. Heavy rains accompanying a hurricane in August reduced the salinity to a low of 14 parts per thousand, but no observations were made at the time of low salinity. The sea water used for preparation of the suspensions was aged sea water and plankton-free. It had a salinity of 35 parts per thousand. The scallops were not subjected to any significant change in salinity during the tests.

Observations were made at room temperature, which was held relatively constant throughout the summer and fall. The experimental suspensions ranged from 21.9° to 25.8° C. Variations during any one period of observation averaged 0.50° C., and only twice exceeded 1.0°.

Single species cultures of a diatom, *Nitzschia closterium* (56 microns in length), and a flagellate, a species of *Chlamydomonas* (7 microns in size), were made radioactive from the incorporation of radioactive phosphorus,[2] P^{32}, following the methods developed at our laboratory (Rice, 1953).

It is not difficult to culture many species of phytoplankton so that each cell is highly radioactive. These cells, when grown in enriched sea water containing virtually no phosphorus excepting the carrier-free radiophosphorus, will take up large amounts of the isotope. This isotope is a useful one emitting only β particles of rather high energy and very measurable. Since phosphorus is one of the major nutrients required, the P^{32} will be incorporated rapidly into organic compounds with little remaining in the exchangeable phosphorus of the cells. With rapidly dividing cells grown in this way, there is practically no radioactivity outside the cells in the sea-water medium.

[2] Obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

The concentrated phytoplankton cultures, or portions of them, were added to the aged sea water to give the desired cell concentration. The number of cells present after dilution was calculated from measurements of aliquots of the concentrated culture using an improved Neubauer haemocytometer. Changes in the cell concentration of the suspension were followed by radioactivity measurements of aliquots removed at regular intervals. In some experiments samples were taken at various locations in the jar. No significant differences were noted, and the measurements made for each time interval were averaged. Control jars without scallops were sampled in order to measure the rate of settling of the suspensions. Although measurable, settling was so slight as to be negligible. Since the cell population after dilution could be calculated from exact microscope counting, and the radioactivity of the cells accurately measured, any changes in cell population could be detected even though extremely slight. It would be quite impossible to detect such slight changes occurring in short periods of time in dilute suspensions using electrophotometer measurements. In fact the original starting concentration was often lower than that which would give a measurable difference from ordinary sea water blanks using such equipment.

The radioactivity of 10-ml. aliquots of the plankton suspension was measured after the addition of nitric acid to disintegrate the cells and dilution to 25-ml. volumes. A dip type Geiger-Müller (G-M) counting tube was used immersed to a fixed depth in a 50-ml. centrifuge tube and connected to the conventional type scaler. Necessary correction for radioactive decay of the P^{32} was made.

It was originally thought that observations with the G-M tube immersed directly in the suspension would do. However, there was soon an accumulation of radioactive cells around the tube even though it was treated with the non-wetting agent, Desicote. Another objection was the limited time of measurement which might not provide enough counts to give the desired accuracy.

Dilution of the phytoplankton culture with sea water to give the experimental cell concentration was great and very little P^{32} was in the sea water of the suspension. Nearly all the radioactivity was within the cells. However, duplicate aliquots of the suspension were filtered through a freshly-prepared barium sulfate precipitate held on a No. 42 Whatman paper and the small amount of radioactivity of the filtrate averaged and subtracted as an additional background for each observed reading. The preparation of this very retentive filter was described by Rice (1953). Filtration at the end of the observations showed the radioactivity measured was due to the presence of cells.

Results

On being immersed in an experimental phytoplankton suspension, the scallops opened almost immediately and soon started to filter the water through their gills. This resulted in a lowering of the cell content of the suspension. A semi-logarithmic plot of the cell concentrations measured at frequent intervals throughout the period of observations was made to visualize these changes. Figure 1 shows examples of the rate of clearing of suspensions of *Chlamydomonas* and *Nitzschia* cells with different concentrations of cells at the start of the experiments. The rate of clearing varied, but followed the same pattern in nearly all of the tests.

There was a gradual increase in the rate of reduction for a time following the immersion of the scallop. Generally, this took place rather promptly, although in a

few instances the decrease in cell number was delayed slightly. This gradual increase in rate of reduction of phytoplankton cells at the start of the observations would be expected since the experiments were started with immersion in the suspension. It was, of course, necessary for the scallop to adjust itself to the new conditions and resume its filtering activities. There was no delay from failure to open as

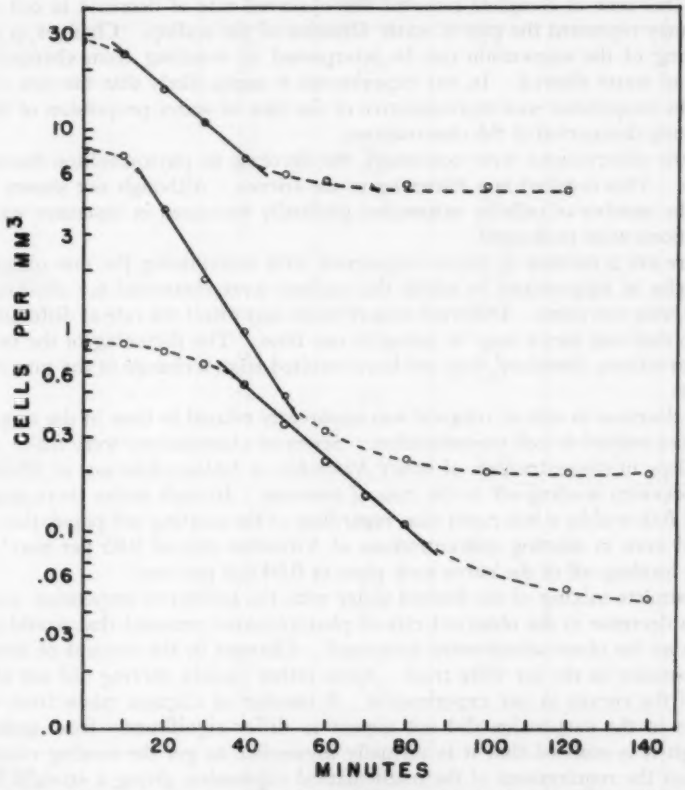

FIGURE 1. Changes in concentration of phytoplankton cells in suspensions with time after immersion of scallops. Upper curve was with use of *Chlamydomonas* cells, lower two with *Nitzschia*. The solid lines represent the calculated regression lines.

might occur in tests using other lamellibranchs. Normally scallops do not remain closed for any long period.

After the initial changes in the rate of particle reduction, a nearly constant percentage decrease of the suspended phytoplankton took place. The regression line for the logarithms of the cell concentrations observed during this decrease was calculated by the method of least squares and drawn for each of the experiments. The solid straight lines of the graphs in Figure 1 represent the calculated regression lines.

The observed points fall very close to these lines. In some instances it was possible to draw a straight line connecting the observed points.

If one assumes that the conditions of the experimental arrangement were satisfactory, the straight line decrease in the logarithm of the cell concentrations with time fits the mathematical equation for a constant rate of water filtration with complete removal of the suspended phytoplankton from the water filtered by the scallop. During this time of complete removal, the observed rate of decrease in cell concentration may represent the rate of water filtration of the scallop. Changes in the rate of clearing of the suspension can be interpreted as resulting from changes in the amount of water filtered. In our experiments it seems likely that the rate of clearing of the suspension was representative of the rate of water propulsion of the scallops during this period of the observations.

As the observations were continued, the decrease in phytoplankton became less and less. This resulted in a flattening of the curves. Although not shown in Figure 1, the number of cells in suspension gradually increased in instances where the observations were prolonged.

There are a number of factors concerned with determining the rate of reduction of particles in suspensions in which the scallops were immersed for observation of their filtering activities. Different ones of these may affect the rate at different times, or more than one factor may be acting at one time. The flattening of the curves of our observations, therefore, may not have resulted from a change in the rate of water filtration.

The decrease in rate of removal was apparently related to time in the suspension. It was not related to cell concentration. Series of observations were made starting the scallops in concentrations of either *Nitzschia* or *Chlamydomonas* at which there was a previous leveling-off in the rate of removal. In each series there was rapid removal followed by a less rapid rate, regardless of the starting cell population. This occurred even in starting concentrations of *Nitzschia* cells of 0.85 per $mm.^3$ and in which a leveling-off of the curve took place at 0.04 cell per $mm.^3$.

Incomplete mixing of the filtered water with the unfiltered suspension would result in a decrease in the observed rate of phytoplankton removal that would get less and less as the observations were continued. Changes in the amount of stirring of the suspension in the jar were tried. Even rather violent stirring did not alter the shape of the curves in our experiments. A number of aliquots taken from various locations in the suspension did not appear to differ significantly from each other. Although it is realized that it is virtually impossible to get the mixing required to fully meet the requirement of the mathematical expression giving a straight line decrease, we believe that recirculation of filtered water through the scallop before mixing was not wholly responsible for the flattening of the curves we observed.

The decrease in the rate of removal reflected in the flattening of the curves could be interpreted as a change in the rate of water propulsion of the scallop. However, such a change was not apparent. The scallops were seen to be creating a considerable current of water even when there was no appreciable decrease in suspended plankton. This was also true when the cell concentration of the suspensions increased from a return of phytoplankton cells after earlier removal. It seems not unlikely that the change in the rate of clearing of the suspension was a result of a changed efficiency in the filtering by the scallop, together with a return of cells previously entrapped in the mucus of the gills.

TABLE I

Water filtration rates of scallops

Range in shell length (mm.)	Weight of meats (gm.)	Starting concentration (cells/mm.³)	Liters/hour filtered		Rate per scallop (l./hr.)	Rate per gram (l./hr.)
			Nitzschia suspensions	Chlamydomonas suspensions		
38–44		20		2.3		
		21	4.0			
		23	1.0			
		23		1.9		
		32		3.5		
		32	4.1			
		44	3.4			
		50		5.9		
		56		5.5		
		57		1.0		
Average	3.3		3.13	3.35	3.26	0.99
47–48		23	13.8			
		34	7.8			
		38		6.5		
		44		9.9		
		78	8.0			
		85	3.3			
Average	8.8		8.22	8.20	8.21	0.93
54–56		2.2	14.7			
		4.7	19.3			
		6.4		10.5		
		8.6		13.3		
		11	5.6			
		21	6.7			
		27		3.5		
		31		8.6		
		33		9.0		
		57	8.5			
Average	12.7		10.96	8.98	9.97	0.79
64–65		0.85	9.8			
		1.0	21.6			
		7.9	25.4			
		22	14.0			
		22	16.3			
		22	13.6			
		22	21.5			
		34		6.8		
		38	5.5			
		52	16.6			
		59	10.8			
Average	20.6		15.51		14.72	0.71

If one assumes that the scallop was filtering the suspension efficiently with complete removal of the suspended particles, it is possible to calculate the rate of water propulsion by the application of the formula given by Jørgensen (1943). This is

$$conc_t = conc_0 \cdot e^{-\frac{m}{M} \cdot t}, \text{ or } m = \frac{(\log conc_0 - \log conc_t) \cdot M}{\log e \cdot t}$$

in which M is the volume of suspension in liters, m is the quantity of water moved in liters per hour, while $conc_0$ and $conc_t$ are the cell concentrations at the initial time and after t hours of time. Undoubtedly the rates obtained are not absolute. They can only represent an approximation since the original assumption made is not likely to be entirely correct. Escape of the plankton through the gills, if occurring as a fixed percentage of the concentration in the suspension, would not change the nature of the curve but would alter the slope. The measured pumping rate may actually be lower than the true rate.

In our observations we can well assume that the changes in cell concentration in the suspensions represent efficient filtration with complete removal of the suspended cells by the gills excepting for the latter part of the observation times when flattening of the curves took place. We have therefore calculated the rate of water propulsion for the scallops for that time after adjustment to the immersion during which a semi-logarithmic decrease in suspended phytoplankton cells occurred. The rates for observations of different sized scallops immersed in suspensions of *Nitzschia* or *Chlamydomonas* at the various concentrations tested are shown in Table I.

Individual scallops in observations made at different times differed in their rate of filtration, but under like conditions of test the rates were reasonably uniform. The average rates were the same for experiments using either species of phytoplankton for the suspended material. They did not appear to be related to concentration. As the scallops increased in size, their rate of water filtration increased. In June and early July the small scallops averaged about 3 liters per hour. Later during the summer they filtered increasing amounts, and by fall were pumping an average of nearly 15 liters per hour. The maximum rate observed was 25.4 liters per hour for a scallop measuring 65 mm. in length. We did not determine the rate of filtration of the scallops in late fall or early winter when the average size of this year class was somewhat greater.

Although the number of observations was small, the average rate of water filtration per gram of tissue was greater for the smaller scallops than it was for the larger. It is not known what effect the development of the gonads in the late summer may have had on the rate of filtration.

Discussion

The use of radioactive plankton cells for determination of changes in the concentration of cells in suspensions in which a filter-feeding animal is placed is valuable for studies of the filtering activities of these animals. Since the cells can be made very radioactive, it is possible to use very dilute suspensions. It eliminates the need for growing large quantities of plankton in culture for such experiments. The suspensions can be made more comparable to normal sea-water environments. Even in a large volume of suspension in relation to the animal under observation, repeated

frequent sampling with the withdrawal of small aliquots is possible. The accuracy of determining radioactivity in extremely minute amounts allows very accurate estimation of cell numbers. No attempts were made in the present study to explore the limits of determination of changes in cell concentrations, nor how few cells in suspension could be measured. However, the radioactivity of the plankton cells used could have been increased considerably, and larger aliquots could have been taken. In all the experiments the aliquots were diluted for radioactive counting and a counter with a rather thick wall (30 mg./cm.^{2}) was used.

The rates of water filtration were measured in cell concentrations well below those that were found by Loosanoff and Engle (1947) to interfere with the pumping activities of oysters. The concentrations used were within those that the scallop might encounter in nature. A short review of the literature on the estimation of phytoplankton abundance in the sea is given by Bainbridge (1953). Galtsoff (1949) presents a discussion of blooming of plankton in the sea. He reports the number of *Gymnodinium* in the surface water in a red tide off Florida as reaching a concentration of 56 cells per mm.^{3}. Cole (1939) measured the phytoplankton content of the sea water pumped into his experimental oyster tanks, and Gaarder and Spärck (1932), Alvik (1934), and Gaarder (1938) report observations for Norwegian oyster pools and fjords. Their values range from 4 to 24 cells per mm.^{3}. Recently, in Great Pond, Massachusetts, a tidal estuary, Hulburt (personal communication) observed a range of phytoplankton from 1.8 to 24 cells per mm.^{3} in carefully counted samples through different seasons. For more open waters, Riley (1941) states that the range he found on Georges Bank from September, 1939 to June, 1940 was from 0.15 to 85 phytoplankton cells per mm.^{3}. Except for most unusual situations, diatoms generally are not as abundant as flagellates and smaller nannoplankton. The importance of nannoplankton in the food-chain cycles of the sea has now become recognized. It is likely that the normal population of diatoms in the sea is about 0.5 cell per mm.^{3} (Hardy and Gunther, 1935). It can be seen that the cell concentrations used in our experiments may not be excessively high, with the possible exception of some in which the diatom *Nitzschia* was employed.

Although the indirect method of measuring the rate of water filtration of lamellibranch molluscs has been used in a number of studies, only in two papers has detailed information as to the changes in the rate of particle reduction during the time of observation been given. Fox, Sverdrup and Cunningham (1937) show data indicating a semi-logarithmic decrease in suspended calcium carbonate with time of observation in experiments using the California mussel. Such a decrease indicates complete removal of the suspended material from the water filtered by the mussels and a rate of filtration relatively constant for the time of measurement. They did observe, however, in certain preliminary experiments a gradual decrease in the rate of reduction with time. This did not take place in later experiments when adequate stirring was provided to minimize the effect of recirculation of filtered water through the mussels.

Jørgensen (1949a) compared the rate of water filtration of the mussel, *Mytilus edulis*, when immersed in suspensions prepared with colloidal graphite, the diatom *Nitzschia*, and three species of flagellates. Changes in the concentration of the suspensions were determined photometrically. The rates of filtration, calculated on the reduction of suspended material, varied with the type of particle used and often

changed during the period of observation. In his experiments using graphite particles, there was a gradual decrease in the rate of clearing; the rate at the beginning of the experiments was greater than at the end. In a few observations in which the phytoplankton constituted the suspended material, there was also a decrease in the rate of clearing of the suspensions. For most experiments using plankton cells, however, he found the rates constant or increasing during the tests. In many instances he reports the rate at the end as being greater than at the start.

In our observations on the rate of clearing of suspended phytoplankton cells by the scallop we found, as did Jørgensen for *Mytilus*, an increasing rate of clearing after the start of observations. This was followed by a time during which the rate was relatively constant. However, in our experiments, as we continued the observations further there was a lessening of the rate of removal of phytoplankton cells similar to the change observed by Jørgensen when suspended graphite particles were used. Later, if the observations were still further continued, there was an increase in the number of cells in suspension.

The pelecypods are able to change the efficiency of their gills as a filtering organ. They form a mucous coat over the gill surface which results in efficient particle retention. The importance of the mucous coating in the feeding activity of lamellibranchs has been stressed by MacGinitie (1941). There is also a possible change in pore size by regulation of the ostea of the gills. Galtsoff (1928) observed that a great number of bacteria added to the sea water were not retained by the gills of the oyster. Similar findings of variations in the retention of bacteria by oysters were reported in more recent work using millipore filters (Galtsoff and Arcisz, 1953). Loosanoff and Engle (1947) observed great variations in the numbers of *Chlorella, Nitzschia,* and *Euglena* removed by the gills of oysters at different times. Jørgensen (1949a) interprets the changes he observed in the clearing of graphite suspensions by *Mytilus* as probably due to the change in efficiency of the gill with a stopping of the secretion of mucus if the particles in suspension were unsuitable for food or the animal disturbed. The efficient removal of the phytoplankton cells was explained in the light of an entrapment in the mucous sheet. Jørgensen and Goldberg (1953) present data showing that the graphite particles used in their experiments were retained almost completely in the filtering organs of the lamellibranchs and the ascidians tested.

In our experiments with the scallop, it seems that the changes in the rate of clearing of the suspensions of phytoplankton cells probably were directly related to the efficiency of the gills. There was an apparent complete removal of the cells from the filtered water after the scallops had become adjusted to the conditions of the experiment. It seems likely that there was formation of a mucous coat which allowed complete retention of the cells in the gills. As the experiments progressed, there was a return from the mucus to the suspension of cells previously removed. This return took place in increasing amount. There may also have been a lessening in the efficiency of the gills in removing suspended cells. It would be unwise to ignore entirely the possibility that the conditions of experimental arrangement had no influence on the observed changes in the rate of clearing of the suspensions. It is almost impossible to have the exact mixing of the filtered water with the unfiltered suspension required to present the scallop with the properly diluted suspension each moment.

The filtration rate may not indicate a true feeding rate, for removal of the cells from the suspension may not result in ingestion by the scallop. Considerable work remains to be done on the role of the mucous coat in filtration and feeding activities. The factors concerned with changes in the secretion of mucus and those governing sorting for ingestion of filtered material need further investigating.

The complete removal of the suspended plankton cells from the water filtered by the scallop allows the use of measurements of the rate of particle reduction to serve as a means of calculating the rate of water propulsion. This indirect method is valuable when methods of direct measurement of the flow are not feasible.

There were no apparent differences in the rate of water filtration of the scallops when immersed in suspensions of phytoplankton cells of different concentrations. Loosanoff and Engle (1947) found that the water filtration of the oyster was reduced when exposed to heavy suspensions of micro-organisms. They observed no effects, however, when the concentrations were light and more comparable to those which may be expected to occur in natural marine waters. Although the threshold concentration for effect varied with the size of the species employed, the values found were high. They reported that oysters were able to reduce the population of plankton to concentrations below this threshold rather rapidly in small containers and then were able to clear the suspension normally. It is conceivable that the concentrations used in our experiments exceeded the effective concentration threshold that would interfere with the rate of water filtration of the scallops and the small volume of the suspensions used enabled the scallops to reduce the cell numbers rapidly to a point below this threshold. If such were the case, we would have failed to observe the effects of concentrations above this threshold on the rate of water filtration. It would be possible to increase the volume of the experimental suspension greatly and provide adequate mixing. The scallops then would reduce the population of plankton cells more slowly and be exposed to high concentrations for greater times. The use of radioactive plankton would still allow the measurement of slight changes in cell numbers. It would be interesting to observe if thresholds of cell concentrations affecting the filtration rate existed that were within those concentrations that the scallop might encounter in nature. In low densities the rates were not markedly increased or decreased over those in higher concentrations. This is in agreement with Jørgensen's observation (Jørgensen, 1952) that filtration rates were not influenced by low concentrations of available food in lamellibranchs and ascidians.

In our observations the scallops were found to have a rather high rate of water propulsion. This rate was greater for the larger scallops than for the smaller. Although the large scallops pumped greater quantities of water through the gills than did the small ones, the average pumping rate per gram of tissue in the larger was somewhat less. Our observations were based on a single year class followed as they grew to an age of 12 to 14 months. During this time there was, of course, development of ripe gametes, which may influence the rate as measured for the larger scallops, causing it to be somewhat high. Similar relationships of rate of pumping to size were reported for mussels by Jørgensen (1943, 1949a), Fox, Sverdrup and Cunningham (1937), Willemsen (1952), and Rao (1953).

If we consider the data available, it appears that the adult oyster (*Crassostrea virginica*) of about 4 or 5 years of age at summer temperatures probably has a water propulsion rate of about 12 to 14 liters per hour (Galtsoff, Chipman, Engle and

Calderwood, 1947; Loosanoff and Nomejko, 1946; and Jørgensen, 1952). Although actual data are not available, the oysters used probably weighed somewhat more than 12 to 14 grams. This would mean that the oyster has a rate of water propulsion a little less than one liter per hour per gram of body weight. This is about the rate observed by us for the bay scallop. For the large mussel, *Mytilus californianus*, Rao (1953) shows data indicating that at 20° C. the animals from 30 to 70 grams had a pumping rate of 5 to 6 liters per hour, and a rate per gram of only 0.1 and 0.2 liter per hour. Evidently this animal has a much lower rate of water propulsion than either the bay scallop or oyster. For *Mytilus edulis*, the rates per animal for small individuals at 22° were about 0.16 liter per hour and for larger from 0.3 to 0.4 liter per hour (Jørgensen, 1949a). For large individuals (70 to 80 mm. in length), at temperatures from 11.8 to 14.7° C. an average of 1.8 liters per hour was found by Willemsen (1952). It seems likely that *Mytilus edulis* may have a rate similar to that of *Mytilus californianus*. The cockle, *Cardium edule*, was found by Willemsen (1952) to have a rate of about 0.5 liter per hour for individuals of 30 to 40 mm. in length at temperatures from 17.3 to 19.5° C. This would indicate that sea mussels and cockles have a rate of water propulsion per gram much less than either the bay scallop or oyster.

Jørgensen (1952) has related the filtration rates of several filter-feeding invertebrates to their oxygen consumption. According to his findings there is a quite uniform ratio between the two in all such forms. In view of the fact that the bay scallop has a rapid rate of growth and is a very active animal, it is not surprising that the rate of water filtration is rather high. The oxygen consumption has not been determined, but it is likely to be also high, and may have the same relation to filtration rate that Jørgensen found for related animals.

Summary

1. The water filtration by the bay scallop, *Pecten irradians*, was studied by following the clearing of suspensions of plankton cells that had been made radioactive. The use of radioactivity measurement techniques for such studies of the rate of water propulsion of filter-feeding invertebrates by the indirect method allows detection of slight changes in cell numbers in dilute suspensions and is advantageous for investigation of the feeding activities of lamellibranch molluscs.

2. The scallops were observed to filter the water passed through their gills very efficiently with apparently complete retention of *Chlamydomonas* and *Nitzschia* cells after adjustment to the immersion in the suspensions. The rapid rate of decrease of suspended plankton was not continued, however, for there was evidence of a decrease in the efficiency of the gills and increasing return to the suspension of phytoplankton cells previously removed.

3. The bay scallop has a relatively high rate of water propulsion, probably correlated with its rapid rate of growth and active mode of life. The average rate for small scallops, 38–44 mm. in length, was 3.26 liters per hour. The largest scallops, about 12 to 14 months of age and measuring 64–65 mm. in length, averaged 14.72 liters per hour. The maximum rate observed was 25.4 liters per hour. The smaller scallops had a rate of about one liter per hour per gram of tissue, whereas the older scallops pumped an average of about 0.7 liter per hour per gram.

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ON THE DISTRIBUTION OF A MARINE CLADOCERAN, PENILIA AVIROSTRIS DANA (CRUSTACEA, BRANCHIOPODA), WITH A NOTE ON ITS REPORTED BIOLUMINESCENCE

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The marine cladoceran, *Penilia avirostris* Dana, is a member of the surface plankton found in warm and temperate coastal waters at widely scattered points throughout the world. Its recent regular appearance in the waters around Woods Hole, Massachusetts, far to the north of its previously supposed temperature limits, has prompted the writing of the present paper. From a survey of the literature it was found that the reported distribution of *Penilia* poses a number of problems of exceptional interest. These problems are here discussed, and the results are reported of certain observations made on living specimens of *Penilia* at Woods Hole. No very definite conclusions have been reached, but it is hoped that other investigators will continue the study, since *Penilia* would appear to be an organism offering certain special advantages to the student of plankton distribution.

Colonization of the Sea from Fresh Water

Of all planktonic marine organisms *Penilia* is perhaps the easiest to identify, for it is the sole marine representative of the cladoceran tribe Ctenopoda, characterized by six similar pairs of filter-feeding trunk appendages enclosed by a bivalve carapace. Indeed even in the related tribe Anomopoda (in which the trunk appendages are not all alike) there are no marine representatives, other than *Bosmina maritima,* found only in the Baltic. Two other cladoceran genera, *Podon* and *Evadne,* are marine, but are members of the aberrant family Polyphemidae, in which the much reduced carapace does not cover the somewhat leg-like trunk appendages. At the present time only a single species of *Penilia* is recognized (see Steuer, 1933a), so that the task of picking *Penilia* out from a plankton sample can be assigned to anyone capable of using a microscope.

Among the many hundred species of filter-feeding Branchiopoda, *Penilia* alone is truly marine. Yet in the other classes of free-swimming Entomostraca, namely the Branchiura, Ostracoda, and Copepoda, we find a much more even distribution of species between marine and fresh water habitats. One might infer that perhaps it is the possession of leaf-like trunk limbs, or "phyllopodia," which has tended to exclude Branchiopoda from marine environments. Storch (1925) and others have pointed out that the effective shape of phyllopodia, and indeed of other parts of the body in these animals, is dependent on the pressure of the blood in the hemocoel. In a fresh water environment this pressure, or state of turgor, can be maintained by a simple osmotic inflow of water. In salt water, maintenance of the turgor would

be more difficult. Despite this fact, however, there are a few species, in each of the four orders of Branchiopoda (Anostraca, Notostraca, Conchostraca, and Cladocera), which have colonized saline pools (see, for example: Mathias, 1937; Packard, 1883, p. 369; Parenzan, 1932). In the case of the anostracan, *Artemia,* Kuenen (1939) has shown that the blood is always hypotonic to the surrounding saline medium; the turgor of the blood in this animal has to be maintained by an active transfer of water against the osmotic gradient; if the animal becomes unhealthy it soon loses its plump appearance and shrinks in volume. In the case of the cladoceran, *Daphnia magna,* which can be acclimatized to quite high salinities, the blood is kept hypertonic to the surrounding medium, thus favoring an osmotic inflow of water (Krogh, 1939).

Although thus able to withstand high salinities, these Branchiopoda which can live in saline pools have never been successful in colonizing the sea. They are adapted to life in shallow, often temporary waters, and seem unable to survive in the very different conditions met with in the ocean.

The ancestor of *Penilia* must have migrated from fresh water to the sea through some favorable river estuary. Its closest relatives, including especially the genus *Sida* (see Lochhead, 1936–7), live chiefly in lakes rather than in small pools. As in other filter-feeding Branchiopoda, the shape of the phyllopodia and other parts of the body in *Penilia* doubtless depends on the maintenance of internal turgor. Specimens of *Penilia* which have sunk to the bottom of a container have a bedraggled appearance, in contrast to the plump specimens still swimming actively in the water above. It is not known whether the internal turgor is achieved by an active transport of water, as in *Artemia,* or by keeping the blood hypertonic, as in *Daphnia.* Fox (1952) reports that *Penilia,* in common with other small Crustacea, constantly ingests water, through both the mouth and the anus. Much of the ingested water seems to be absorbed, but Fox doubts that this is the primary mechanism for maintenance of internal turgor.

Podon and *Evadne* do not have phyllopodia, their limbs being more like those of ostracods. It may be that the shape of these animals is not so dependent on internal turgor as in other Branchiopoda. In this connection it is perhaps significant that in the family Polyphemidae, to which *Podon* and *Evadne* belong, there are more species in salt than in fresh water.

Penilia, Podon and *Evadne* share in common two features of the reproductive system that are not found in a majority of other Cladocera. Perhaps, therefore, these are features that in some way have proved helpful in the colonization of the sea, though in just what way is not very evident. The fertilized, resting eggs have thick shells, and are not enclosed in a protective "ephippium." The eggs which develop parthenogenetically are small, almost devoid of yolk, and during embryonic development increase greatly in size by absorption of a nutritive fluid secreted by the mother into the brood sac. This last feature is characteristic of both fresh water and marine Polyphemidae, but it is not known for any Ctenopoda other than *Penilia* (Storch, 1925; Sudler, 1899). Perhaps it is no more than a coincidence that the same feature is found also in the anomopod genus *Moina,* species of which are among the Cladocera most frequently encountered in saline pools.

Sporadic and Discontinuous Distribution

Information available to date indicates that *Penilia* has an extremely sporadic and discontinuous distribution. It has been recorded from the shores of every continent, frequently in tremendous numbers, but from widely separated points and often only during a single season. At Beaufort, North Carolina, Sudler found *Penilia* in June, 1896, in such numbers as to clog a net towed for only a few meters through the water. Not until 1931 was *Penilia* again recorded from our Atlantic coast, when it was taken off New Jersey (Bigelow and Sears, 1939). Dr. R. E. Coker very kindly informs me that he later found a single specimen in plankton taken outside Beaufort Inlet, in January, 1949, and that a number of specimens were taken there towards the end of 1953. At Naples, Italy, *Penilia* was unknown until 1922, when Caroli found it throughout the summer, the population becoming so large in late August as to form practically the entire surface plankton. In this case the population became established, and *Penilia* has since reappeared at Naples in large numbers each summer (Cattley and Harding, 1949). In the waters off Sydney, Australia, *Penilia* has been recorded on various occasions since 1894, and recently its volume there was so great (0.85 ml./m.^{3}) as to constitute the richest haul of non-salp plankton taken in Australian waters (Sheard, 1949). This compares with the best hauls of non-salp zooplankton commonly taken in temperate northern waters, although volumes of over 5 ml./m.^{3} have been recorded (Clarke and Bishop, 1948).

Further examples could be cited, but enough has been said to show how suddenly *Penilia* may appear, and the abundance it may attain. The periods of abundance are always associated with a high rate of ovoviviparous parthenogenesis, as in other Cladocera, but the fluctuations must be correlated with as yet unknown environmental factors. Since *Penilia* can be so easily identified, has a direct development uncomplicated by larval stages, and has feeding habits that are reasonably well known (Lochhead, 1936–7), it should be a favorable species for a study of factors underlying such seasonal fluctuations in abundance as are discussed by Sears and Clarke (1940).

An incomplete summary of the discontinuous world distribution of *Penilia* has been given by di Caporiacco (1938). Additional records will be found in some of the references listed at the end of the present paper. No simple explanation is evident for this discontinuous distribution, nor for the sporadic appearances of *Penilia*. In the case of fresh water Cladocera, examples of the same kind can be explained by assuming a chance distribution of resting eggs, especially on the feet of birds, to isolated bodies of water. Coastal waters of the oceans are, however, continuous, and opportunity for the resting eggs of *Penilia* to become attached to the feet of birds would not appear to be great. The resting eggs of Ctenopoda and Polyphemidae are generally said to be cast loose into the water. Weismann (1877) found those of fresh water species lying at the bottom of aquaria. It also has been supposed that the resting eggs of *Penilia, Podon,* and *Evadne* sink in sea water (Bigelow and Sears, 1939; Friedrich, 1952). However, Jorgensen (1933–4) found the resting eggs of *Evadne nordmanni* in surface plankton, enclosed in the parental exuviae. She suggests that they may not sink as rapidly as had been supposed.

They might then be dispersed widely by currents, in spray by the wind, and to beaches where they could be picked up on the feet of birds. It seems unlikely, however, that such a condition holds true for *Penilia*. Caroli (1924), who followed throughout a summer a population of *Penilia* with a relatively high output of resting eggs, never saw these eggs enclosed within exuviae. He concluded that the eggs probably sink to the bottom. Possibly the sudden appearance of *Penilia* in new localities occurs only when a few recently released resting eggs, that have not yet had time to sink, chance to have been caught up in wind-driven spray.

More difficult to explain is the apparent absence of *Penilia* from many intermediate locations. This might seem to indicate that *Penilia* has only recently invaded the sea. If that were true, however, one would expect to find *Penilia* especially abundant in river estuaries, whereas it more usually is found in open coastal waters. Perhaps unsuspected environmental factors have excluded *Penilia* from certain areas. Or it may be that populations in some areas have died out during parthenogenetic reproduction through the action of dominant or semi-dominant lethal genes. Instances of this kind have been recorded by Banta and Wood (1939) for some fresh water Cladocera. Banta (1939) notes that there are wild populations of certain fresh water Cladocera which are never known to reproduce other than by ovoviviparous parthenogenesis. In one case he found a mutant stock in which both resting and "summer" eggs were produced by parthenogenesis. It may well be that in some areas *Penilia* reproduces only by parthenogenesis, probably then without the production of resting eggs. Information on this question is very scanty, since most of the reports dealing with *Penilia* fail to mention the sexes taken or the type of reproduction observed. Steuer (1933a, 1933b) stated that males were at that time known only from Hong Kong, Naples, the Adriatic, and the Black Sea. In the literature I have found only one additional record that seems to refer to the occurrence of males. Dakin and Colefax (1940) state that off Sydney, Australia, *Penilia* was "found breeding in April and May." Since this was at the end of the season, and maximum numbers of *Penilia* were noted in January and February, it seems probable that the statement was meant to refer to bisexual reproduction. One more locality for the occurrence of males and resting eggs can now be added, namely, Woods Hole, Massachusetts, where in August, 1953, I found about one or two per cent of the population of *Penilia* to be males, and a lower percentage to be females bearing resting eggs.

Obviously, further records are needed before any conclusions can be drawn as to the prevalence of males and resting eggs in different localities. It would be helpful if those who record the presence of *Penilia* would always take care to state what types of individual were found. Steuer (1933b) figures juvenile instars and the three types of adult, namely, males, females with one or two resting eggs, and parthenogenetic females with "summer" eggs or embryos. "Summer" eggs which have not yet developed into embryos are much smaller than resting eggs, and in most cases are more numerous. Persons not having access to Steuer's paper should be able to identify the different types of adult by reference to Figure 1, which shows a female bearing a single resting egg, and a male one instar prior to maturity. Mature males have penes over twice the length of those shown, and antennules reaching to the posterior border of the carapace. Males two instars prior to maturity can be recognized

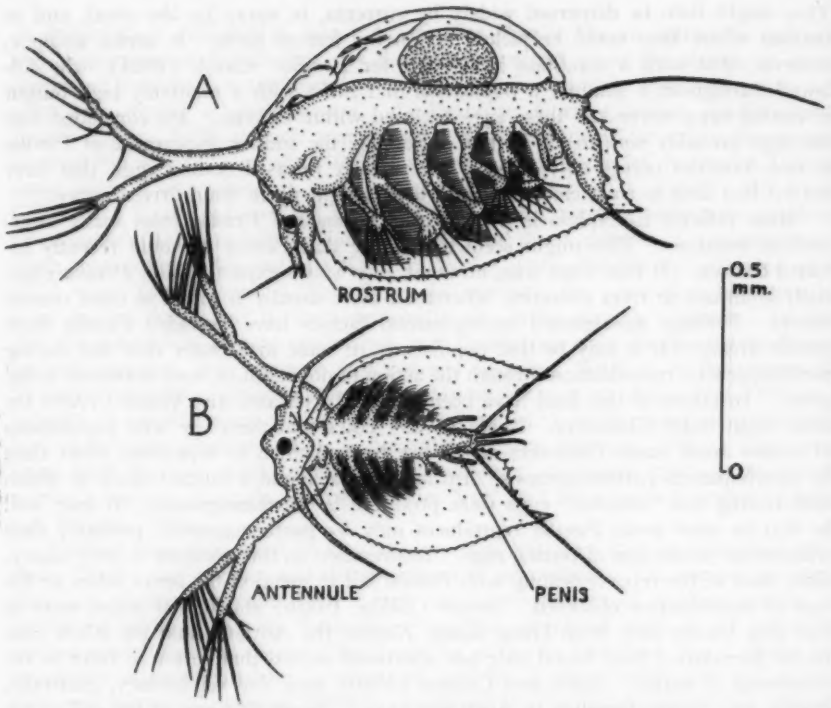

FIGURE 1. *Penilia avirostris* Dana, from Woods Hole, Massachusetts, August, 1953. Sketched from preserved specimens, a camera lucida being used for the main outlines. A, mature female, with a single resting egg in the brood sac; B, male one instar prior to maturity; the shell valves are here much wider apart than they would be in a living specimen.

by small rudiments of the penes, antennules already twice the length of those in a female, and, as in all males, by the absence of a pointed rostrum.

RESTRICTION TO COASTAL WATERS

The restriction of *Penilia* to coastal waters is another feature for which no definite explanation can be offered at present. The greatest distances from shore at which *Penilia* has been taken seem to be those reported by Bigelow and Sears (1939) (about 60 km. off the coast of New Jersey) and by Marukawa (1921) (either about 230 km. or about 310 km. east of Japan). Neither temperature nor salinity would appear to be a factor excluding *Penilia* from the open sea. Some authors have spoken of *Penilia* as a predominantly brackish water form (*e.g.*, Gibitz, 1922). There has even been a single isolated record of *Penilia* from fresh water (Krämer, 1895). However, most of the records of *Penilia* have been from localities with salinities above 32‰. Leder (1915) found *Penilia* in salinities from 10‰

to 37‰. Della Croce (1952) found maximum numbers of *Penilia* in the Gulf of Genoa when the salinity was above 38.3‰. Gurney (1927–9) found *Penilia* abundant in the Gulf of Suez where the salinity was 43.8‰, and even recorded it from a station in the Suez Canal where the salinity was 49‰ (salinities taken from Fox, 1926–9). It is evident that the salinity of the open ocean lies well within the range normally tolerated by *Penilia*. At low temperatures, however, the resistance to high salinities may be reduced, in accordance with Moore and Kitching's (1939) "idea of modified tolerance." Thus Dakin and Colefax (1940) found *Penilia* four miles out to sea off Sydney, Australia, reaching maximum abundance in summer, yet also found it to be sometimes exceedingly abundant in winter in the more estuarine catches. This is despite the fact that minimum surface water temperatures were lower in the estuary (12.3° C.) than at the offshore station (15–16° C.) (Dakin and Colefax, 1935). Salinity at the offshore station was 35.4–35.8‰; that within the estuary was unfortunately not given.

Perhaps the restriction of *Penilia* to coastal waters is a result of a shortage in the open sea of fine detritus and minute organisms, under 5 μ in diameter, on which *Penilia* feeds (Lochhead, 1936–7). However, other animals requiring equally fine food do live successfully in the open ocean.

If it should be found that populations of *Penilia* must everywhere be renewed each year from resting eggs, and if these eggs sink to the bottom, as seems probable, then the restriction of *Penilia* to waters of moderate depth would be understandable. However, in tropical regions one would expect at least some populations to be successful in reproducing throughout the year exclusively by ovoviviparous parthenogenesis.

Restriction to Surface Waters

Apparently *Penilia* always occurs relatively close to the surface. Gamulin (1948–9) records it from depths of 20–70 meters in the Adriatic; Motoda and Anraku (1952) found it only at depths of 10–20 meters in "Funka Bay," Hokkaido, Japan (apparently a synonym for Volcano Bay, judging from a map (Fig. 4) in Kawasaki *et al.*, 1952–3). Nearly all other records seem to have been from surface plankton tows.

I have tested the reactions to light of specimens of *Penilia* taken at Woods Hole, with purely negative results. When illuminated only from one side by a bright, horizontal beam of light from a spot lamp, *Penilia* continued to swim in various directions in all parts of a jar, whereas copepods in the same jar quickly aggregated towards the light. Specimens of *Penilia* also swam at random, both upwards and downwards, when illuminated only from above; the spot lamp was placed 23 cm. above a small tube, holding a column of water 60 mm. high and 18 mm. in diameter, standing on a dull black surface. Copepods tested under these same conditions aggregated immediately towards either the top or the bottom of the tube, depending on the species, in contrast to *Penilia* which showed no such tendency even over periods of up to fifteen minutes. In both the horizontal and the vertical beams of light results were the same with light-adapted *Penilia* and with *Penilia* which had been kept in total darkness for periods of from one to twelve hours. In all cases the specimens spent much time floating suspended in the water, swimming in sudden spurts at irregular intervals, as is characteristic for

the species. Neither when floating nor when actively swimming could the specimens be seen to show any type of dorsal light response to the direction of illumination. It may be concluded that the restriction of *Penilia* to surface waters would appear not to be in response to light.

Nor does it seem that this restriction depends on a response to pressure, of the type discovered in crab larvae by Hardy and Bainbridge (1951). To test for such a response, specimens of *Penilia* which had been kept in the dark for twelve hours were transferred to a 10-ml. syringe, over the lower opening of which a piece of wax paper had been tightly tied. The level of sea water was adjusted up to the mark and the plunger then very slowly pushed down until all air had been expelled. With the lower end of the syringe resting on a table, pressure on the plunger was now increased steadily by hand up to a maximum later found equal to about 10.5 kilograms. Since the internal diameter of the syringe was 14 mm., it can be computed that the maximum water pressure attained, additional to atmospheric pressure, was approximately 6.8 kg./cm.^{2}, corresponding to a depth in the sea of about 67 meters. The specimens of *Penilia* in the syringe were observed against a brightly lighted dark background. At all pressures they continued to swim normally, showing no tendency to aggregate at any particular level in the column of water.

It must remain for the future to determine what factors may be responsible for the restriction of *Penilia* to surface waters. Perhaps a response to temperature is involved, or perhaps under some conditions the animals display negative geotaxis. However, no evident responses to gravity were seen in the specimens observed in vertical tubes.

Distribution as Related to Temperature

The distribution of *Penilia* towards the poles is limited by temperature. Calman (1917–23) stated that the mean annual surface isotherms of 18° C. would include all localities where *Penilia* had been found, except for Trieste, the Black Sea, and possibly New Zealand. This statement remained unchallenged for many years, and it therefore was with considerable surprise that in 1944 I recognized what appeared to be a drawing of *Penilia* among some sketches of plankton organisms turned in by a student in the Invertebrate course of the Marine Biological Laboratory, Woods Hole, Massachusetts. The specimen was dead when found, and had been thrown away after being drawn. In 1946 a plankton sample collected for the Invertebrate course contained considerable numbers of *Penilia,* which I was able to examine alive. The same was true in 1948, 1949, 1951, 1952, and 1953. The samples each year were collected from the surface waters of Great Harbor, Woods Hole, between August 23rd and August 31st, with additional samples in 1953 from July 29th to September 1st. No especial search for *Penilia* was made in any year, so that it is quite likely that *Penilia* has been present in Woods Hole waters each year since 1944. Mr. A. Fleminger, a graduate student at Harvard University, has kindly turned over to me some specimens of *Penilia* taken in surface plankton from Menemsha Bight, Martha's Vineyard, about 20 km. S.S.W. of Woods Hole, July 25, 1951 (one specimen) and September 1, 1951 (25 specimens). Deevey (1952a, 1952b) has recorded the presence of *Penilia* in Block Island Sound, about 90 km. W.S.W. of Woods Hole, in 1943, 1944, 1945, and 1949.

One of her hauls in September, 1943, yielded about 2900 *Penilia* per fifteen-minute tow; another haul in October, 1949, yielded 900 *Penilia* per cubic meter.

Thus it is evident that at least since 1943 *Penilia* has occurred, often in large numbers, off the southern coast of New England. It must be a recent arrival, since it is not mentioned in any of the plankton surveys of the region made prior to 1943. As already noted, most of the specimens seen by me have been females, reproducing when adult by ovoviviparous parthenogenesis. One large specimen contained 13 embryos, a number which exceeds both the observed and the predicted maxima given by Steuer (1933b).

To gain more precise knowledge of the lower temperature limits controlling the distribution of *Penilia*, I have gathered together the information given in Table I. In my search of the literature I have not been able to find any records of *Penilia* from localities farther north or farther south than those shown in this table. It will be seen at once that the mean annual surface temperature at Woods Hole lies far below the limit of 18° proposed by Calman, or the limit of 17° more recently proposed by Fuller (1950). However, it will also be seen that *Penilia* has been taken from four other localities with mean annual surface temperatures well below these limits.

Actually, as pointed out by Hutchins (1947), the poleward distribution of marine organisms is controlled, not by the mean annual surface temperature, but by the lowest temperature tolerated in winter and by the minimum temperature necessary for reproduction in summer. Neither of these temperature limits is known for *Penilia*. Fuller (1950) thought that *Penilia* requires a temperature of at least 18–20° during a period long enough to permit reproduction. This suggestion agrees well with most of the records that we have for *Penilia*. However, Cattley and Harding (1949) found *Penilia* reproducing in October off the coast of Holland, in water of 16.68°. Possibly on occasion even lower temperatures would suffice, judging from the statement of Dakin and Colefax (1940) that in estuarine waters near Sydney, Australia, *Penilia* sometimes becomes exceedingly abundant in winter, when surface water temperatures of about 12–15° might be expected (Dakin and Colefax, 1935).

At Woods Hole the surface water temperatures in summer should more than suffice for the reproduction of *Penilia* (means for July, August, and September in the period 1945–1951: 21.3°, 21.8°, and 20.0°). The temperatures encountered in winter, however, are much lower at Woods Hole than in most of the other localities from which *Penilia* has been recorded. Reference to Table I will show that only in three of the other localities are the surface water temperatures in winter nearly as low as at Woods Hole.

In regions where the population of *Penilia* has been followed through a season, it is known that during the autumn the numbers gradually decrease to zero. The lowest temperature at which a surviving adult has been taken appears to be 8.7° (Steuer, 1933b). Usually all specimens are gone at temperatures well above that value. Survival in colder temperatures presumably must be in the form of resting eggs.

Of the localities listed in Table I, only Woods Hole, Odessa, and Auckland represent regions where *Penilia* has become well established. There are three reports of its occurrence in northern Japanese waters. The other localities represent simply isolated records which could perhaps be looked on as abnormal oc-

TABLE I

Locality	*Penilia* reported by	Mean surf. water temps. (° C.)			Authority for temperatures
		Coldest month	Warmest month	Annual	
Woods Hole, 1945–1951	Present paper	Feb., 0.7°	Aug., 21.8°	11.5°	1
Woods Hole, 35 years of period 1885–1941	No report	Feb., −0.2°	Aug., 21.1°	10.4°	2
Lat. 40°N., 175 km. south of Woods Hole	No report	March, 6.4°	Aug., 20.6°	13.0°	3
Dutch coast, 52° 22′N., 4° 20′E.	Cattley and Harding	Feb., 3.5°	Aug., 17.5°	10.2°	4
Near Odessa, Black Sea	Zagorowsky (see Steuer, 1933b)	Feb., 1.1°	July–Aug., 22.2°	13.5°	3
Muroran, Hokkaido, Japan	Tanita, Kato, and Okuda	March, 2.2°	Aug., 21.7°	11.0°	3
Near Cape Town, S. Africa, 35° 13′S., 20° 17′W.	Rammner	July, 15.2°	Jan., 20.6°	18.2°	3
Cook Strait, New Zealand	Steuer, 1933a	July, 10.0°	Feb., 16.1°	13.2°	5
Auckland, New Zealand	Krämer. Fuller.	July, 12.1°	Feb., 21.0°	16.7°	6

Authorities for temperatures in Table I

1. Calculated from monthly mean surface temperatures in the years 1945–1951 at the Woods Hole Oceanographic Institution dock, consulted through the kindness of Mr. Dean F. Bumpus. Closely similar figures for the years 1945–1950 have been published by the U. S. Coast and Geodetic Survey (Publ. No. TW-1, 1951).

2. Calculated from monthly and annual mean surface temperature records, very kindly supplied by Mr. John B. Colton, U. S. Fish and Wildlife Service, Woods Hole. Records are not available for the period 1915–1932, nor for a few other scattered years.

3. Figures for each month read from: World Atlas of Sea Surface Temperatures, H.O. No. 225, Hydrographic Office, U. S. Navy, Washington, D. C.: 50 p., 1944.

4. Figures for each month read from: Böhnecke, G., and G. Dietrich, 1951. Monatskarten der Oberflächentemperatur für die Nord- und Ostsee und die angrenzenden Gewässer. Deutsches Hydrographisches Institut, Hamburg: 18 p.

5. Figures for each month read from charts in: Monthly sea surface temperatures of Australian and New Zealand waters; Marine Branch Meteorol. Office, Air Ministry, London; M.O.M. 482: 14 p., 1945.

6. Calculated from monthly mean surface temperatures for each of the years ending April, 1929, to April, 1941, in: Report on Fisheries, New Zealand Marine Dept., years ending March, 1931, to March, 1941.

currences. With this in mind it might appear that only where the mean surface temperature in the warmest month reaches or exceeds 21° can *Penilia* become permanently established. Perhaps such warm temperatures are necessary to permit the building up of a minimal population density, sufficient to assure the survival of at least some individuals, or resting eggs, through the winter.

In localities such as Woods Hole and Odessa, where temperatures are adequate in summer but very low in winter, one must consider the possibility of repopulation each spring from warmer waters to the south. This probably would have to be by currents, if we accept the assumption, presented earlier, that resting eggs can be caught up in wind-driven spray only when newly released, which would be chiefly in late summer and autumn. Unfortunately our knowledge of non-tidal surface currents south of the New England coast is very incomplete. The complexity of the situation is well presented by Haight (1942). More recent information is given by Zinn (1950), and an analysis of the complex tidal currents near Woods Hole has been presented by Redfield (1953). Rather detailed charts of non-tidal surface currents in the waters east of the United States were published for each month of the year by the German "Oberkommando der Kriegsmarine" (1943), but must include much that is based only on conjecture. In general, it would seem that coastal organisms to the south of New York would stand little chance of being brought to New England waters; organisms from the Gulf Stream, directly to the south of New England, such as the siphonophore *Physalia*, certainly are brought to the New England coast in some summers, but the existence every year of such currents from the south is highly doubtful.

Table I shows that if *Penilia* occurred just beyond the continental shelf to the south of Woods Hole, it would encounter minimal winter temperatures of about 6.4°. But summer temperatures there might not be adequate for a high rate of reproduction; the presence of *Penilia* so far from land would not be expected; and the annual occurrence of suitable currents to bring the organisms to the coast seems rather unlikely.

Thus it appears probable that *Penilia* is now a year-round inhabitant of Woods Hole waters. If so, the resting eggs must be able to withstand winter temperatures down to below zero (the mean surface temperature at Woods Hole for January, 1948, was $-1.2°$; a temperature of $-2.8°$ was recorded on one occasion in 1945). In effect this would mean that the only temperature requirement for *Penilia* is a summer temperature of perhaps 21° or above, with lower temperatures possible in regions where a high population density is not necessary to assure survival through the winter.

From Table I it will be seen that water temperatures at Woods Hole in the period 1945–1951 averaged slightly higher than in previous years. However, the facts stated above make it unlikely that the slightly colder winters of the past were responsible for the absence of *Penilia* from New England waters.

Discussion

It must remain for future investigation to provide more certain data, and in particular to discover an explanation for the apparent absence of *Penilia* from so many regions that would appear favorable for its development. Doubtless its occurrence has frequently been overlooked, but in regions where the plankton has been inten-

sively studied one would expect it to be reported if present. Thus at both Woods Hole and Naples, *Penilia* is now abundant, but would seem to have been absent at least for many years in the past.

Possibly such sudden inroads into new areas have been preceded by a process of genetical pre-adaptation to wider temperature limits, as reported for fresh water Cladocera by Brown (1929). (See also Johnson, 1952.) Perhaps, too, the production of resting eggs by *Penilia* occurs only in a few genetic stocks, of localized distribution. Reproduction by parthenogenesis alone might lead to local extinction, as noted earlier, and the failure to produce resting eggs presumably would prevent colonization of areas where water temperatures are low in winter. Many other interesting possibilities could be suggested. The whole question of how the population cycle of *Penilia* may be related to the population cycles of other planktonic organisms remains virtually an untouched field. Some data relative to this problem will be found in the papers of Dakin and Colefax (1933), Deevey (1952b), Massutí Alzamora (1949), and Sheard (1949).

Tests for Bioluminescence

A report concerning *Penilia* that would be of considerable interest if it could be confirmed is contained in the paper of Dakin and Colefax (1940), who state that *Penilia* "appears to be one cause of luminescence in our plankton." They add that "no distinct luminous organs have been noticed." In August, 1953, I attempted to repeat Dakin and Colefax's observation, with wholly negative results. Dark-adapted specimens of *Penilia* were picked out from a freshly caught plankton sample, and were observed in the dark in groups of ten or more concentrated in a small volume of water. Stimuli used to provoke luminescence were mechanical stirring with a glass rod, drop by drop addition of formalin, ammonium hydroxide, alcohol, and glycerine, the make and break passage of an electric current at various intensities from an inductorium, and exposure to ammonia vapor, the specimens in this case being supported in air on a piece of bolting silk. These stimuli include the principal ones used by Giesbrecht (1895) in his search for luminescent copepods. In no case did any of the specimens of *Penilia* exhibit bioluminescence. This was in contrast to other unidentified microscopic organisms, which luminesced brightly in samples of the plankton from which all specimens of *Penilia* had been carefully removed.

It seems reasonably certain that *Penilia* is not luminescent at Woods Hole in the month of August. However, Giesbrecht found that copepods at Naples, if they luminesce at all, do so for the most part only in the winter. Dakin and Colefax observed *Penilia* in Australian waters at all times of the year, and perhaps it was in the winter that their specimens seemed to be luminescent. If *Penilia* is self-luminous at times, it is probably unique in this respect within the class Branchiopoda (Harvey, 1952), and it might be cited as an especially interesting example of the general rule that among aquatic organisms the only ones that are luminescent are marine.

Summary

1. *Penilia* has recently become a regular member of the summer plankton off the southern coast of New England, much farther north than would formerly have been expected.

2. Its reproduction in New England waters is mainly by ovoviviparous parthenogenesis, but males also occur, together with females bearing fertilized, resting eggs. There is reason to believe that the species overwinters locally in the form of resting eggs, rather than being brought in each year by wind or currents.

3. The poleward distribution of *Penilia* seems to be limited only by the need of a certain minimum water temperature in summer. Adults can survive at temperatures down to below 9° C., and are able to reproduce at and perhaps below 16° C. But the species has not become permanently established except in areas where summer water temperatures exceed 21° C. The resting eggs probably can survive winter temperatures of below − 2° C.

4. *Penilia* is found in every ocean of the world, often as one of the dominant plankton organisms, yet it is absent from many regions where it might be expected to thrive. Possible reasons for this discontinuous distribution are discussed. Sudden appearances of *Penilia* in new areas perhaps are the result of recently laid, resting eggs becoming caught up in wind-driven spray.

5. The restriction of *Penilia* to coastal waters may be because the resting eggs sink to the bottom. Salinity is not the limiting factor, since *Penilia* has been found in salinities ranging from fresh water to 49‰.

6. *Penilia* shows no obvious responses to light or to pressure which might account for its restriction to surface waters.

7. The fact that *Penilia* is the only filter-feeding branchiopodan to have become truly marine, may possibly be because in sea water it is difficult for members of this group to maintain the necessary turgor within their phyllopodia.

8. A report that *Penilia* is bioluminescent could not be confirmed for specimens examined in August at Woods Hole, Massachusetts. The possibility remains that in some localities it may be luminescent in winter.

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THE FIBRILLAR SYSTEMS OF CILIATES AS REVEALED BY THE ELECTRON MICROSCOPE. II. TETRAHYMENA [1]

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Recently the fibrillar systems of ciliates have attracted the attention of several investigators. This revival of interest has resulted in large measure from an appreciation of the morphogenetic properties of these systems. These properties appear to include self-duplication, genetic continuity, pluripotency and a role in the establishment of the polarity and fine morphology of ciliates (Fauré-Fremiet, 1948a; Lwoff, 1950; Weisz, 1951). Unfortunately, continued progress of these studies has been limited by an inadequate understanding of the micro-structure of ciliate fibrillar systems. However, a clearer knowledge of these structures has recently been obtained from an electron microscope study of *Paramecium* (Metz, Pitelka and Westfall, 1953). The present investigation was undertaken to extend this study to other material.

Tetrahymena was selected for this second effort not only because of the wide popularity of this organism as a laboratory animal but also because it can be cultured readily in the absence of other organisms; its fibrillar morphology has been studied exhaustively within the limits imposed by light optics (most recently by Corliss, 1952a, 1953); its morphogenesis at fission has been examined in detail (Chatton, Lwoff, Lwoff and Monod, 1931; Furgason, 1940); and finally its locomotion appears to involve an acetylcholine-acetylcholinesterase system (Seaman, 1951; Seaman and Houlihan, 1951).

The pellicle and oral anatomy of *Tetrahymena* are found to differ greatly from that of *Paramecium* but the kinety (silver line or neuromotor) systems of the two organisms are basically similar in structure and organization. The kineties of *Tetrahymena*, like those of *Paramecium*, are compound structures composed of discrete units. Each unit consists of a cilium, a kinetosome (ciliary basal body) and a short, tapering, kinetodesmal fibril with periodic structure. As reported briefly elsewhere (Metz, 1953), these units are associated by overlapping of the individual kinetodesmal fibrils to form the kinetodesma or silver line fiber of the light microscopist.

Material and Methods

Tetrahymena geleii,[3] strain W, was used exclusively in this investigation and the writers are indebted to Dr. G. W. Kidder for the initial culture of this organism. All subsequent cultures were grown in 2% proteose peptone in the absence of other organisms. Three- to seven-day old cultures served as starting material for electron

[1] This investigation was aided by a grant from the American Cancer Society.

[2] Present address: Department of Zoology, Florida State University, Tallahassee, Florida.

[3] Following an exhaustive study Corliss (1952b) concludes that the specific name *pyriformis* should have priority over *geleii* (Furgason, 1940).

microscope examination. With few exceptions these rich cultures revealed no dividing animals when examined with light optics.

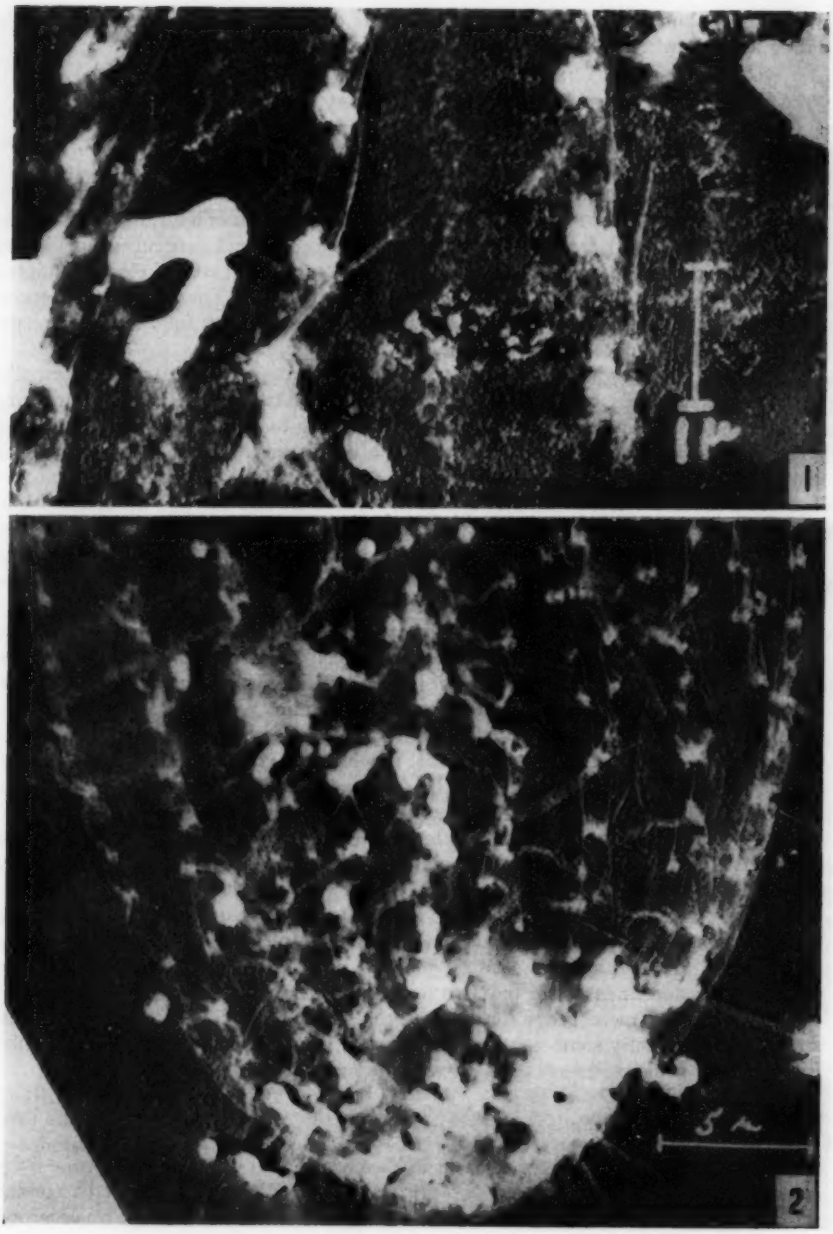
Preparation of the material for electron microscopy

The preparation procedure employed in the *Paramecium* study (Metz, Pitelka and Westfall, 1953) was transferred to *Tetrahymena* with little modification. It consisted of the following steps: (1) fixation, (2) distilled water washing to remove fixative, (3) sonic dissection (9 Kc magnetostriction oscillator, model S-102A, Raytheon Corp.), (4) fractional centrifugation to isolate and concentrate pellicular fragments, (5) mounting (air drying specimens to collodion-coated electron microscope screens) and (6) shadow casting with chromium. The critical dissection, concentration and mounting operations (steps 3, 4 and 5) were followed with phase optics.

Formalin and osmium tetroxide vapor were employed as fixatives. Formalin fixation was achieved by mixing equal volumes of untreated *Tetrahymena* culture and diluted formalin (commercial formalin diluted to 1/15 with distilled water giving a final formalin concentration of 3.3% in the mixture). The mixture was allowed to stand for one to several hours and finally processed as described above. To obtain osmium tetroxide fixation, one to several ml. of *Tetrahymena* culture (with or without previous concentration by centrifugation) were poured into a syracuse or petri dish and exposed to osmium tetroxide vapor in a closed vessel. Following fixation the material was processed as outlined above. The osmium tetroxide material was examined most extensively. Although this method of preparation sometimes gave beautiful preparations, it did not do so consistently. In many cases the fibrillar apparatus appeared to be coated with precipitated cytoplasm and mitochondria when examined with electron optics. Indeed the kineties of *Tetrahymena* appeared to serve as preferential sites for such precipitation and in a number of preparations the primary kinety fibrils were completely obscured by such material. This difficulty was encountered in other forms, notably *Blepharisma* and *Stentor*, in preliminary examinations. This variable behavior may have an important bearing upon the results obtained with the silver impregnation technique (*e.g.*, von Gelei, 1935; Corliss, 1952a). With limited access to the electron microscope it was not possible to standardize the procedure to the point where consistently favorable results were obtained. However, fixation appears to be the critical operation. Prolonged treatment with osmium tetroxide vapor is to be avoided. Just sufficient exposure to kill the organisms, followed by judicious sonic treatment (phase microscope observations at 30-second to one-minute intervals), gave the most satisfactory preparations. Indeed the best specimens were fixed so lightly that the cilia frayed out (Figs. 1, 2) and macronuclear membranes sometimes broke down revealing interesting internal structure. Evidently some fixation is necessary, for all attempts to obtain pellicular fragments by sonic treatment of living animals produced only breis.

Electron microscope examination

The material was examined with an RCA model EMU electron microscope using standard procedures. However, one unusual detail of procedure must be emphasized. This concerns the orientation of the specimen in the electron microscope.



FIGURES 1–2.

The specimens are fragments of the protozoan surface, they are asymmetrical (three axes of asymmetry, *e.g.* Figs. 1, 6) and their relations to the morphology of the intact animal must be interpreted in terms of this asymmetry. The specimen screen may be mounted in the electron microscope so that the specimen side of the screen faces toward or away from the photographic plate of the instrument and either arrangement is acceptable practice. However, a reversal of symmetry will result in changing from one situation to the other. When the specimen surface of the screen faces away from the plate, normal symmetry relations obtain on the plate; when the specimen surface faces toward the plate, normal symmetry is reversed and a mirror image results. This problem is seldom critical in electron microscopy (Hillier, personal communication) but its importance cannot be over-emphasized in the present instance.[4] It follows that all subsequent photographic manipulations (reversal or "second negative" and final printing) must be performed with equal attention to symmetry.

The screens were always mounted specimen side down in the electron microscope specimen screen holder (facing the photographic plate). Proper orientation was facilitated by using Athene screens (Smethurst High-Light Limited) which have easily recognizable differences in their surfaces.

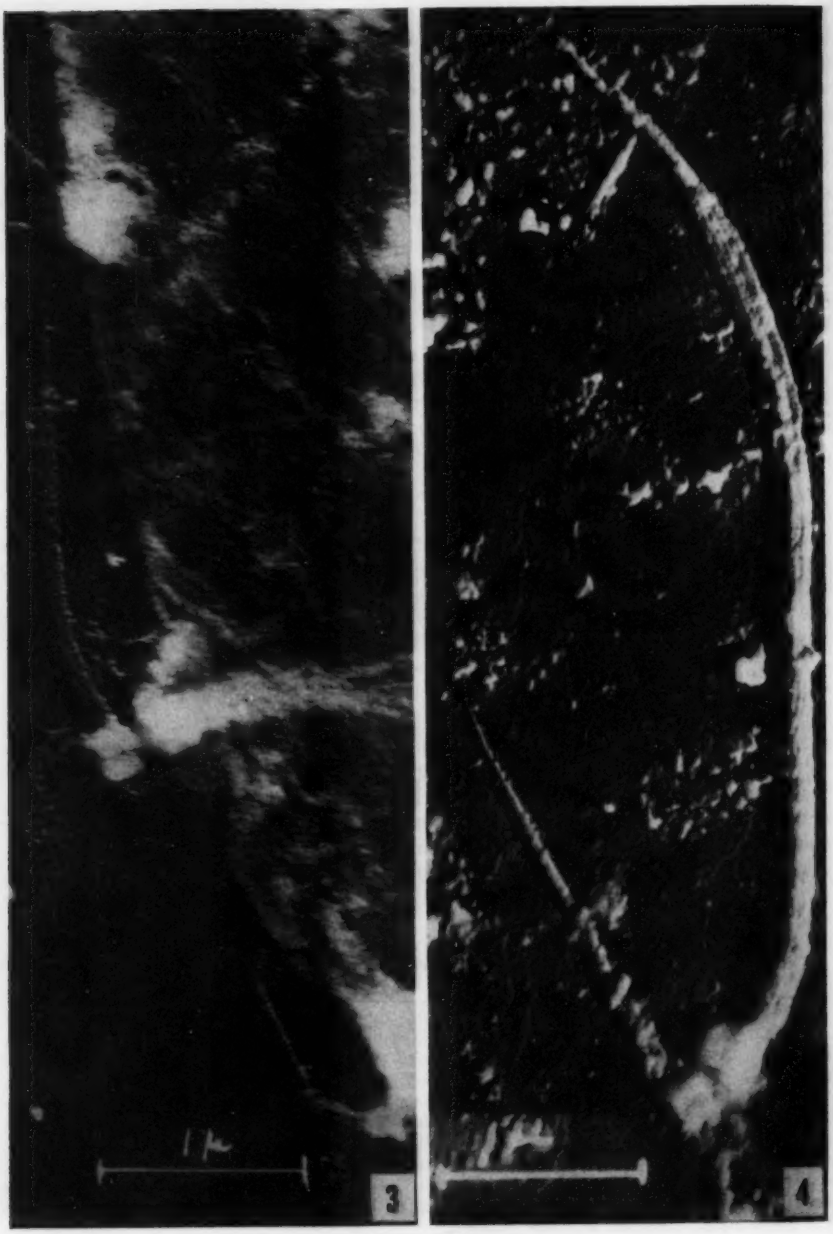
Limitations of the method

The rather drastic methods of preparation employed introduce certain hazards and limitations to the study. These include the following: (1) artifacts may be introduced by fixation, sonication and drying; (2) observations are usually made on fragments of many organisms selected at random, making it difficult to reconstruct the morphology of a single individual; (3) the sonic dissection method involves removal of parts of the organism under conditions that preclude direct observation. These limitations have been discussed and evaluated in the previous study (Metz, Pitelka and Westfall, 1953). They apply with nearly equal force in the present instance. However, *Tetrahymena* does possess two striking advantages over *Paramecium*. First, when grown in the absence of other organisms, all material observed with the electron microscope may be considered to be a part of the organism or a byproduct of it, if suitable precautions are taken to prevent organic and other contamination during preparation. Second, *Tetrahymena* is of such a size that large, easily recognizable fragments or whole, "eviscerated" animals can be examined with the electron microscope (*e.g.*, Fig. 2). Both of these advantages were exploited in this investigation.

[4] These precautions were followed in the Paramecium study although they were not stressed in that paper.

FIGURE 1. A fragment of pellicle viewed from the inner surface. Segments of three kineties are shown. Each of these consists of several kinety units (3, 4 and 5 units in the right, middle and left kineties, respectively). Each kinetodesmal fibril is seen to arise from a kinetosome and overlap the next unit. Bases of the cilia pass through ring-shaped thickenings in the pellicle. The striated area of the pellicle is seen immediately to the right of the kineties. The chains of ring-shaped thickenings in the pellicle are evident midway between the kineties. The granular area of the pellicle is located to the right of the pellicular chains. Part of a frayed cilium is visible through the pellicle (upper left). Osmium tetroxide fixation.

FIGURE 2. Posterior end of an "eviscerated" animal. The individual kinetodesmal fibrils are directed toward the anterior end of the animal. Frayed cilia may be seen on the margins of the preparation. Osmium tetroxide fixation.



FIGURES 3–4.

Results

When properly prepared by osmium tetroxide fixation the material contains fragments of pellicle of various sizes (Figs. 1–3), the isolated oral apparatus (Figs. 5, 6) and formed bodies of the cytoplasm (mitochondria, macronuclei). The units of kinety structure generally remain attached to the pellicular fragments in osmium tetroxide-fixed material but they may be obtained in isolated form (Fig. 4) from formalin-fixed animals. Since the electron photomicrographs show interesting details of structure in the pellicle, kinety system and oral apparatus, these will be considered separately.

A. Structure of the pellicle

The pellicle is a thin membrane with three structurally distinct regions. These are most evident in Figure 1. This figure shows that the inner surface of the pellicle consists of (1) a "background area" of rather uniform granular appearance, (2) a "chain" of ringlike thickenings parallel to and midway between the kineties and (3) a finely striated area immediately to the right of each kinety.

Background area

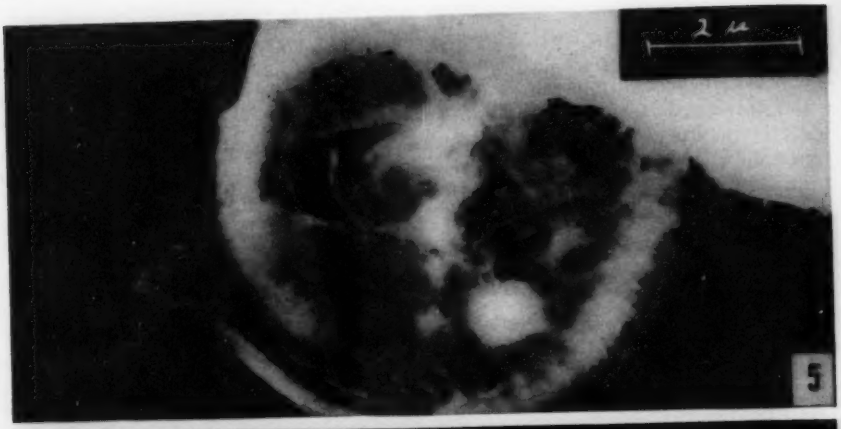
This region warrants little comment. The granular appearance of this material may be ascribed to macromolecular components of the inner surface of the membrane or to sub-pellicular protoplasmic components which have remained attached to the membrane proper. Study of this region at high magnifications and with special techniques might reveal further interesting details, possibly comparable to those of the erythrocyte membrane (Hillier and Hoffman, 1953).

Chains of ring-like thickenings

The chains of ring-like thickenings are the most striking structural feature of the pellicle. Two systems of such chains appear to be present in *Tetrahymena*. The first and more prominent of these occurs with great regularity and consists of chains located midway between, and parallel to, the kineties. The second, less evident system parallels both the first system and the kineties and is located at the level of the kineties. This second system is found in many but not all preparations but it may occur generally, for this region of the pellicle is frequently obscured to a greater or less extent by the kinetosomes, kinetodesmal fibrils and cilia. The

Figure 3. "Simulated cross section." The pellicular fragment has folded upon itself. The fold forms the left margin of the specimen, the outer surfaces of the pellicle are opposed, with the frayed cilium from the central kinety unit sandwiched between these surfaces. The fold is oblique to the axis of the animal. Therefore the three prominent kinety units belong to three different kineties. The chains of ring-shaped thickenings run diagonally from lower right to upper left in the upper layer of the pellicle. They pass around the fold to the lower layer of pellicle and proceed from lower left to upper right, thereby forming an angle of approximately 90° with the parent row in the upper layer. The two systems of ring-shaped chains may be seen in this preparation. One of these is located at the level of the kineties, the other runs midway between kineties. Osmium tetroxide fixation.

Figure 4. The isolated unit of kinety structure. The long cilium consists of a narrow terminal portion, the main shaft with indications of longitudinal fibrils and the basal bulb. This bulb is to be distinguished from the kinetosome situated just below the bulb (compare with Fig. 3). The kinetodesmal fibrils which arise from the kinetosome again show periodicity. Formalin fixation.



FIGURES 5–6.

first system but not the second is evident in Figures 1 and 2. Both systems are present in Figure 3 (see caption to Fig. 3 for details).

Although the chains of ring-shaped thickenings occur with great regularity, their form is somewhat variable. This variability may be ascribed in part to variations in fixation. The rings are usually more prominent following prolonged fixation (osmium tetroxide) and their centers frequently appear more transparent in such preparations. Other variations can not be ascribed to fixation and may result in part from uneven stretching of these thin membranes during drying. In its simplest form the chain is a single even row of closely spaced rings (Fig. 2) but this construction, while not uncommon, is sometimes modified so that the chains consist of bands two or more rings wide (Fig. 1). In other preparations the chains may branch or the rings may be widely scattered in the granular area of the pellicle. However, these last variations are exceptional.

The striated area

The delicate striated areas are apparent only in favorable preparations but in these they occur regularly and are constant in position and form (Fig. 1). These areas are situated immediately to the right of each kinety.[5] Each consists of a narrow (0.25 μ wide) band of material which runs parallel to the adjacent kinety and to the long axis of the animal. The characteristic feature of these bands is the collection of fine, closely spaced ridges (fibers?) that run lengthwise along the bands. The origin, composition and function of these bands are unknown. They are assumed to be part of the pellicle proper because of their constancy of occurrence and form. However, the possibility that they consist of sub-pellicular material adhering to the pellicle has not been excluded.

B. The kinety system

It will be recalled (Metz, Pitelka and Westfall, 1953) that the kineties of *Paramecium* are compound structures. They are built up of discrete units. Each of these units consists of a cilium, a kinestosome (ciliary basal body) and a relatively short, tapering, kinetodesmal fibril. These units are associated by their kinetodesmal fibrils. The fibrils overlap in shingle-like fashion to form a bundle,

[5] "Right" as employed here refers to the animal's right. When the pellicle is viewed from within and the figure is oriented with the anterior end of the animal upward (Fig. 1), then the animal's right will correspond to the observer's right. When the fragment is viewed from the outside, with the anterior end upward, the animal's right will be the observer's left.

FIGURE 5. The posterior part of the oral apparatus viewed from its inner surface. The "fan" of parallel fibers is clearly seen to pass to the left and beyond the margin of the oral apparatus from the undulating membrane (membranelle I). At their origins the fibers appear to be in pairs or to have double origins in the lower part of the figure. Traces of the complex systems of fibers which interconnect the membranelles are visible in the upper part of the figure. Osmium tetroxide fixation.

FIGURE 6. The oral apparatus viewed from the outer surface. The base of the undulating or first membranelle passes along the right side (observer's left) of the preparation. A portion of one cilium remains attached to the base of this membranelle. The base of the second membranelle is located on the anterior left margin of the preparation. The third and fourth membranelle bases are located more posteriorly. The fan of fibers from the first membranelle passes to the left and under the left margin of the preparation. These fibers presumably pass down the wall of the cytopharynx in the intact animal. See text for further details. Osmium tetroxide fixation.

the kinetodesma (neuromotor or silver line fiber) of earlier light and electron microscope studies (Bretschneider, 1950; Sedar, 1952). The kineties of *Tetrahymena* are similar in structure to those of *Paramecium* in all important essentials.

The unit of kinety structure

Each kinety of *Tetrahymena* is composed of discrete units. One such unit is shown in Figure 4. This isolated structure is composed of a cilium, a kinetosome and a kinetodesmal fibril. The cilium and kinetodesmal fibril form an angle of approximately ninety degrees as they arise from the kinetosome.

The *cilium* of *Tetrahymena* has the usual structure since it appears to consist of an outer limiting membrane (Fig. 4) enclosing a number of longitudinally arranged fibers. With mild fixation the membrane breaks down and the fibers fray out (Figs. 1, 2, 3). In the intact cilium (Fig. 4) three regions may be distinguished. These include 1) the main shaft, 2) a distal, narrow portion constituting 1/6th of the cilium and 3) a proximal bulb. A similar narrow tip has been described in *Paramecium* (Metz, 1954) but the proximal bulb is evidently unique. This bulb is located at the base of the cilium as the latter structure passes through the pellicle. The proximal ciliary bulb should not be confused with the kinetosome. These two structures are separated by a constriction at the level of the pellicle. The relative positions of the ciliary bulb, pellicle and kinetosome may be determined by comparing Figures 3 and 4. Figure 3 shows a simulated cross section—actually a folded pellicular fragment with the outer pellicular surfaces opposed (see caption to Fig. 3 for details). One kinety unit is located on the margin of the fold and the pellicle is seen to pass between the proximal bulb of the cilium and the kinetosome. The ciliary bulb may be an artifact of preparation. However, its presence in both formalin and osmium tetroxide preparations does not favor this possibility.

The *kinetosome* appears to be a double structure (over-all dimensions 0.2 $\times$ 0.3 μ) consisting of two bodies separated by a constriction perpendicular to the surface in the transverse plane of the animal. One of these bodies, the primary one, appears to be slightly larger than the other and gives origin to both the cilium and the kinetodesmal fibril. The second body forms an appendage to the first, 180° from the site of origin of the kinetodesmal fibril. These relations are evident in Figure 3.

The *kinetodesmal fibrils* arise from the kinetosomes and run just below and parallel to the pellicle (Figs. 1, 2, 3). These structures gradually taper to a fine point from a base diameter of approximately 0.12 μ (calculated from Figs. 3 and 4; it is assumed that the specimens have suffered no appreciable flattening since they cast well proportioned shadows). Calculations from several preparations indicate a length of 1.5 to 2 microns for the fibrils. The average length of fibrils in Figure 1 is 1.5 μ; lengths in Figures 3 and 4 are 2.1 microns. The kinetodesmal fibrils show an interesting periodicity in favorable shadow-cast preparations (especially clear in Fig. 3). This periodic organization appears as a series of transverse ridges separated by constrictions in the preparations so far examined, but further study may resolve these into spiral ridges similar to those of the *Paramecium* kinetodesmal fibrils (Metz, Pitelka and Westfall, 1953).

Association of the units to form kineties

In *Tetrahymena* as in *Paramecium*, the units of kinety structure do not appear to exist as unrelated, randomly arranged, isolates. They are organized to form

larger structural (and probably functional) units, the kineties. The units are arranged in longitudinal rows with the kinetosomes and cilia spaced at regular intervals (Figs. 1 and 2). The individual kinetodesmal fibrils are directed along these longitudinal rows and since they are longer than the distance between kinetosomes, they overlap in shingle-like fashion (Fig. 1). This overlap associates one kinety unit with the next adjacent unit. The series of overlapping fibrils constitutes the kinetodesma of the light microscopist. This construction is essentially the same as that found in *Paramecium*. In *Tetrahymena*, however, the kinetodesmal fibrils are so short (less than two interciliary distances) that true bundles of fibrils are never formed. A cross section of a kinety at any level should not show segments of more than two fibrils. In *Paramecium* the maximum number appears to be five.

In life the association of the fibrils in the region of overlap is presumably an intimate one and it appears to be so in some electron photomicrographs (Fig. 2), but in many preparations (Figs. 1, 3) the fibrils are widely separated at the region of overlap. This may be ascribed to the violent action of the sonic treatment, surface tension forces during drying and the relatively small area of contact between the fibrils. This failure to observe intimate association in most cases serves to emphasize the apparent absence of any sheath or cementing material binding the fibrils together. Indeed, no information is yet available concerning the details of this association. It is one of the more interesting problems arising from the study.

Polarity

Examination of the figures reveals that the kinetodesmal fibrils of the unit structures all extend in the same direction from their kinetosomes. They are polarized. Therefore, it becomes a matter of some interest to establish the direction of this polarity; to determine whether the fibrils extend toward the anterior or posterior end of the animal. Three independent observations bear upon this problem and they show that the fibrils pass toward the anterior end of the animal. These observations are the following: 1) The kinetodesmal fibrils pass to one side of the next adjacent kinetosome (Fig. 1). This arrangement is regular and consistent in all electron photomicrographs except for an occasional fibril. These exceptional cases may be ascribed to alterations of the pattern during preparation. Indeed it is astonishing that the pattern is so regular considering the violent nature of the treatment. This pattern is believed to be the basis for the "rule of desmodexy" (Chatton and Lwoff, 1935; Lwoff, 1950). This rule states that kinetosomes always lie to the left (animal's left) of the kinetodesma. When the photomicrographs are oriented according to desmodexy, the fibrils are found to pass forward, *i.e.*, anteriorly. This relation is illustrated in Figure 1. Since this figure shows the inner surface of the pellicular fragment, the observer's right and left correspond to the animal's right and left. 2) The isolated oral apparatus is highly asymmetrical (Fig. 6) and is easily oriented (see next section) with respect to the anterior-posterior axis of the animal. Occasionally the oral apparatus is isolated with pieces of adjacent pellicle attached and the orientation of the kinetodesmal fibrils attached to such pellicle can be established with respect to the anterior-posterior axis of the neighboring oral apparatus. Nine cases of this sort were examined. In eight of these the fibrils were directed anteriorly. In the 9th the fibrils were directed posteriorly but this may not be a valid case for it is possible

that this figure represents an isolated oral apparatus lying over a fragment of unrelated pellicle. 3) When subjected to mild sonic treatment large segments of individual animals may be obtained. In favorable preparations these are largely free of internal protoplasm. Such "eviscerated" animals may retain the original form of the organism. The posterior end of such a preparation is illustrated in Figure 2. The fact that there are two layers of pellicle, one over the other, renders it difficult to follow some of the kineties and to determine which kinety belongs to which layer, but the figure clearly shows that the individual kinetodesmal fibrils pass anteriorly from their kinetosomes.

C. Oral anatomy

The preparations obtained by sonic dissection contain large numbers of oral structures in various degrees of isolation (see previous section under polarity). Representative examples are shown in Figures 5 and 6. With the exception of the oral cilia these preparations appear to consist of substantially the entire oral apparatus including the four "membranelles" and their interconnecting fiber systems.

The orientation of these isolates is readily established. This may be done by reference to the abundant figures in the literature from light microscope studies, by reversing argument number 2 presented above in the section on polarity, by examining large fragments (argument No. 3 in the above section on polarity) and by phase microscope examination of fixed material preliminary to sonic treatment. Applying these criteria the specimen illustrated in Figure 6 is found to be an outside view of the oral complex and the foundations of three of the four "membranelles" are readily identified. The right (animal's right, observer's left) and about half the posterior margin of the oral complex consist of the first of these, the so called "undulating membrane." A portion of one component cilium remains attached to the foundation of this membrane. The second "membranelle" is readily identified on the left (animal's left, observer's right) anterior margin and the third is located immediately posterior and nearly parallel to the second. The fourth "membranelle" is almost certainly situated in the electron-opaque material posterior to the third "membranelle," where a number of kinetosomes appear to be located. However, this region has not been observed clearly in the outside views of the oral apparatus and it is equally obscure in inside views. These complexes and their interconnecting fiber systems invite the detailed descriptions given below.

Membranelle No. 1 (undulating membrane)

The foundations of the membranelles are relatively dense and reveal less detail than might be desired. The first membranelle is no exception. It appears to consist of a marginal row of cilium bases, presumably kinetosomes. Segmentally arranged masses of material, 0.8 μ long, extend "medially" (presumably toward the interior of the animal) from these. They appear to correspond in number, but not necessarily in position to the ciliary bases and are readily identified in the anterior portion of the membranelle (Fig. 6). In some figures (Fig. 5) these masses appear to be broken up in a fashion to suggest at least three parallel rows of cilium bases. However, light microscope studies (*e.g.*, Furgason, 1940) show only one row of cilia in this membrane and electron microscope figures of specimens with intact cilia support this view. A suggestion of a longitudinal fiber (or series of

short overlapping fibers?) is visible just medial to the cilium bases in the anterior end of the membranelle foundation (Fig. 6).

Although these details of structure have their interest, the most striking feature of the membranelle is the fan-shaped series of fibers that extend from it. In the figures this fan extends toward the left of the oral apparatus, under the left posterior margin and beyond. This is clearly shown in Figures 5 and 6. The fans of fibers illustrated in these figures are broken off relatively close to their origins. In some other figures, however, they appear to be relatively intact. In these they are found to extend at least 13 microns beyond the left margin of the oral apparatus. The fan of fibers tapers as it extends; whether this taper results from decrease in fiber number or from reduction in fiber diameter (or both) has not been determined, but if there is no reduction in number, the longest fibers must extend for approximately 20 microns (nearly half the length of the animal) from their bases. Presumably these fibers extend down or close to the right side of the gullet in the intact animal, but where they end has not been established.

The relation of these fibers to the kinetosomes of the membranelle cilia presents an interesting challenge to the microanatomist. The individual fibers of the fan arise at or near the kinetosomes of the membranelle. However, there appear to be fewer fibers than kinetosomes (16 and 28, respectively, in Fig. 6). No fibers are seen to extend to the first three or four kinetosomes in Figure 6, whereas more posteriorly a fiber seems to extend to every second kinetosome. The function of these fibers and their relation to other organelles present interesting problems for future study. The fact that some of the fibers appear double in Figure 5 enhances this interest.

Membranelles II, III and IV

The second and third membranelles each appear to consist of at least three rows of ciliary bases or kinetosomes. This is evident only for membranelle III in Figure 6. Figures not reproduced here show a similar condition in membranelle II. The region of membranelle IV has such high electron-scattering power that little detail is revealed in any of the photomicrographs so far obtained. A single row of kinetosomes does extend into this area from the anterior end of membranelle III and the individual kinetosomes of this row appear to be highly constant in position and number.

The four membranelles are interconnected by complex patterns of fibers that are revealed only in inside views of the oral apparatus. Prominent among these is a series of parallel fibers extending from membranelle II to membranelle III and what may be a continuation of this, a similar system extending from membrane III toward membranelle I (undulating membrane) and possibly connecting with the latter. These fibers appear to join with the membranelle kinetosomes. The appearance of these fibers suggests that they lie relatively deep below the oral pellicle. A second system of short, irregularly arranged fibers occupies a more superficial position. Neither of these systems has been studied in detail and they must be examined more thoroughly before an accurate description can be given.

Oral and kinetodesmal fibrils compared

A comparison of the oral fibers (Figs. 5, 6), especially the fan fibers from membranelle I, with the kinetodesmal fibrils (Figs. 1, 2, 3, 4) of the body ciliature im-

mediately reveals that the two types of fibers are quite different in structure. The kinetodesmal fibrils are short and rather uniform in length whereas the oral fibers appear to vary in length from membranelle to membranelle. The fan fibers in particular are very long. Moreover, the construction of the fibers from the two regions is strikingly different. The kinetodesmal fibrils have the marked periodic organization, clearly revealed in Figure 3. No trace of this periodicity appears in any of the figures of the oral fibers. These figures include fibers that are shadowed along, and at right angles to, the fiber axis. Some figures include both kinetodesmal fibrils with clean-cut periodicity and oral fibers lacking such organization. If the oral and kinetodesmal fibers are homologous structures, as would appear to be the case considering the currently accepted view of stomatogenesis, then these differences in organization must reflect specific differences in a single morphogenetic machine. Since this machine must operate fairly close to the macromolecular level, it and its products warrant special attention and more exhaustive study.

Discussion

This study provides new information of two sorts. The first and more significant of these is confirmatory information of a general nature, the second concerns the specific, detailed morphology of *Tetrahymena*.

Confirmatory

The observations reported here confirm the major contribution of the first study in this series. This contribution concerns the structure of the kineties of ciliates. These are now found to be built up of discrete units in *Tetrohymena* as well as in *Paramecium*. Each kinety unit consists of a cilium, kinetosome and a short tapering kinetodesmal fibril. The units are associated by overlap of the kinetodesmal fibrils to form the kineties and they are polarized toward the anterior of the animal in this association. The confirmation of this basic structure in *Tetrahymena* lends some support to the view that this construction may be widespread if not universal among ciliates.

This basic kinety structure has certain interesting implications for the physiology of conduction and coordination and for morphogenesis in ciliates. These aspects have been discussed at some length in the first paper of this series (Metz, Pitelka and Westfall, 1953) and they need not be repeated in detail here. With regard to conduction it is sufficient to emphasize that an acetylcholine-acetylcholine esterase system appears to be involved in the locomotion of *Tetrahymena* (Seaman, 1951; Seaman and Houlihan, 1951). This may operate in "synaptic" transmission from the kinetodesmal fibril of one kinety unit to that of the next unit (Metz, Pitelka and Westfall, 1953). The operation of such a mechanism is more readily visualized in *Tetrahymena* than in *Paramecium* because of the relatively simpler construction in the former organism. In *Tetrahymena* the overlapping kinetodesmal fibrils are so short that any single fibril contacts only the fibrils of the two units immediately adjacent to it (one anterior, the other posterior, to the given unit). Consequently, any coordinating impulse would necessarily pass stepwise from one kinetodesmal fibril to the next adjacent fibril. In *Paramecium* the kinetodesmal fibrils are relatively much longer, so much longer that they form bundles up to five fibrils in cross section.

Orderly transmission here would seem to require some selective agency (Metz, Pitelka and Westfall, 1953).

Because of its relative simplicity the fibrillar system of *Tetrahymena* should be favorable material for morphogenetic studies at the electron microscope level. It should not be difficult to obtain division stages showing multiplication of the kinety units. Even stages in stomatogenesis are not beyond expectation. But until such preparations are obtained, discussion of morphogenesis beyond that already given (Metz, Pitelka and Westfall, 1953) is of no great value.

Specific morphology

The morphology of *Tetrahymena* and related organisms has been studied extensively by a number of investigators using light optics combined with silver staining. Furgason (1940) and Corliss (1952a, 1953) have reviewed this work thoroughly and their accounts will serve as the basis for the following discussion. The electron optical preparations clarify to some extent the confusion that exists regarding the detailed anatomy of the "ciliary meridians" and certain aspects of the oral morphology in *Tetrahymena*. These particular problems will be considered here.

The ciliary meridians, as revealed by light optics and silver impregnation, are complex and somewhat variable structures. The most constant features include the "primary meridians" connecting the ciliary bases in longitudinal rows and "secondary meridians" running parallel to and approximately midway between the primary meridians. In some preparations even a third or tertiary meridional structure is described. Aside from these meridians transverse structures are frequently observed which extend on the left from the primary meridian to the vicinity of the secondary meridian. The fact that the appearance of these structures varies depending upon the extent of silver impregnation, has led some investigators to the view that many of the described structures are artifacts (*e.g.*, von Gelei, 1935). These structures may be "artifacts" in the sense that they do not exist in the living animal as actual fibers; but there must certainly be some specific, physical basis for them.

With the detailed picture of the kineties and associated pellicle now available from the electron photomicrographs the physical basis for certain of the silver structures may be discussed with some confidence. The "primary meridians," at least in heavily impregnated specimens, lie in the region of the overlapping kinetodesmal fibrils. The silver probably precipitates around, over, or in these fibers to give the effect observed in heavily impregnated material. In lightly treated preparations the meridians assume a lateral loop effect to the left between adjacent kinetosomes. The known structure of the system does not readily account for this phenomenon; nor does it account for the lateral extensions from the primary meridians in heavily impregnated material. However, in over-fixed animals prepared for electron microscopy (actually quite lightly fixed), cytoplasmic material appears to precipitate preferentially over the kineties and to extend as lateral projections in a more or less regular fashion (see Methods section). It is possible that such material serves as sites for silver precipitation.

The chains of ring-like thickenings in the pellicle readily account for the "secondary meridians" of light microscopy, assuming that some silver precipitates in the region of these structures. The longitudinally striated area of the pellicle lies to the right of the kineties and apparently remains rather free of silver, although a fine "fiber" is sometimes observed in this region with light optics.

The above account offers explanations for most of the meridional structures described in silver impregnated material. However, it should be recalled that the subpellicular cytoplasm was removed by the method of preparation for electron microscopy so there is ample room for further speculation. Indeed there appear to be many possible explanations for some of the structures observed in silver preparations and it is unlikely that exhausting them here would serve a useful purpose.

The electron photomicrographs of the oral structures reveal the wealth of detail described earlier in this paper. The relation of this detail to the interpretation of light optical studies should be obvious. However, there are two points that deserve further mention.

One of these is the margin of the oral apparatus. This is delimited on the right and part of the posterior edge by the first or "undulating" membranelle and on the left anterior margin by the second membranelle. Elsewhere the margins are free of membranelle structure. In these free regions the marginal pellicle may be somewhat thickened, but it appears more likely that it is supported by the underlying fiber network. Indeed it is likely that the entire oral apparatus is held together in these fixed preparations by the complex, interconnecting fiber system. Whether or not this fiber system serves a supportive function in the living animal is problematical. Nothing suggesting the circumoral ring of light optics appeared in the electron photomicrographs.

The second point of particular interest concerns the broad fan of fibers associated with the first or undulating membranelle. This structure has not been revealed in detail by light optical studies, but the rods, ribs (Corliss, 1953) and "zone intermédiaire" (Fauré-Fremiet, 1948b) that appear in silver preparations certainly represent fragments of the fan fibers. Furthermore, there is no longer any excuse for confusing the individual fibers of the fan with cilia.

Favorable preparations of the pre-oral region have not yet been obtained. The inferior ones that have been examined fail to show the "intermeridional connecting fibers" of light microscopy. Undoubtedly some basis for these in the form of fibers or pellicular sculpturing as well as other interesting morphological details will reward further investigation.

The electron microscope at North Carolina State College was used in this study. We wish to thank Dr. A. C. Menius and Mr. W. T. Withers for its use and for their assistance.

We also wish to thank Dr. D. P. Costello and the members of his department for the hospitality of their laboratories, for their interest in the work and for many helpful suggestions.

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Summary

1. Macronuclei, mitochondria, fragments of the pellicle and the oral apparatus of *Tetrahymena* may be isolated for electron microscopy by sonic dissection of osmium tetroxide fixed material. The fine morphology of the pellicle, the attached fibrillar structures and the oral apparatus are described.

2. The pellicle consists of a thin membrane which is sculptured to give a striated area immediately to the right of, and parallel to, each kinety, and two longitudinal ring-like thickenings, one at the level of and the other midway between the kineties.

3. The kineties of *Tetrahymena*, like those of *Paramecium*, are compound structures built up of discrete units. Each unit consists of three parts:

(a) The cilium which passes through a ring-shaped thickening in the pellicle.
(b) The ciliary basal body or kinetosome. This structure consists of two parts separated by a constriction.
(c) A short, tapering, kinetodesmal fibril which arises from the kinetosome.

These units of kinety structure are associated by their kinetodesmal fibrils. These fibrils overlap in shingle-like fashion to form the kinetodesma of the light microscopist. The individual kinetodesmal fibrils are highly polarized in this association. They all taper toward the anterior end of the animal. This mode of organization has now been demonstrated clearly in two ciliates, *Tetrahymena* and *Paramecium*. It will not be surprising if it is found to occur generally in ciliates.

4. The detailed structure of the oral apparatus, including the bases of the four membranelles and certain of their fibers, is revealed with new clarity. Prominent among the latter is a large fan-shaped group of fibers which arises from the first or undulating membranelle and probably extends deep into the interior of the animal.

5. The physical basis for some of the structures revealed to the light microscopist by silver impregnation is discussed.

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THE UPTAKE AND UTILIZATION OF PHOSPHATE IONS FROM SEA WATER BY THE AMERICAN OYSTER, CRASSOSTREA VIRGINICA (GMEL.) [1]

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Phosphorus is of physiological importance to mollusks not only in their carbohydrate metabolism and energy transfer systems but also in shell deposition. Both Manigault (1939) and Bevelander and Benzer (1948) have associated shell deposition with phosphatase activity, and the latter also suggested that the first material to be deposited is calcium phosphate, which is changed into calcium carbonate in the presence of a phosphatase. Love and Frommhagen (1953) have found further evidence that calcium phosphate is the precursor of calcium carbonate in *Mactra* (*Spisula*) *solidissima*. Bevelander (1952) exposed several species of mollusks to $P^{32}O_4$ ions in sea water and found in radioautographs that P^{32} was localized on the inner surface of the mantle, below the surface epithelium. Since aqueous methods were used in the preparation of the tissues, the P^{32} shown by his radioautographs was only that fraction which had entered water-insoluble combinations.

Two sources of phosphorus are potentially available to marine, ciliary feeding mollusks: that combined in suspended particulate matter, such as plankton and detritus, and that in solution in sea water in various chemical combinations. The latter may be divided into phosphate ions in equilibrium with various cations and dissolved organic compounds which include phosphorus in their makeup.

Ronkin (1950) has shown that $P^{32}O_4$ ions are absorbed by the excised gill of *Mytilus edulis*, and he suggests that some of this phosphorus is used in the production of adenosine triphosphate by the gill, to be used in maintaining the extensive ciliary action there. It has been further shown (Pomeroy and Haskin, 1951) that phosphate ions are absorbed by the oyster from sea water principally through the gills, and that these ions appear rapidly in the blood and eventually in other tissues. This report gives further information on the uptake and distribution of phosphorus in the oyster, the absorptive mechanism, and the importance of the direct utilization of ions from the surrounding water.

Materials and Methods

Phosphorus analyses on sea water were performed by the method of Redfield *et al.* (1937), modified for the use of a photometer. Phosphorus analyses on tissues were made by wet-ashing them in sulfuric and nitric acid, driving off the excess nitric acid by slow heating, carbonization of the organic matter in the remaining sulfuric

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acid, oxidation with 30 per cent hydrogen peroxide, and proceeding as with sea water samples. All tissues were collected and ashed in duplicate. Aliquots from the wet-ashed tissue samples were evaporated to dryness on planchets of uniform area and counts made of their radioactivity using a scaler and counting tube with an end-window density of 1.4 mg./cm.^{2}. Samples were sufficiently small and dilute to prevent appreciable self-absorption. This was verified by counts of a suitable graduated series of sample dilutions.

Radioautographs were made by two of the methods of Holt and Warren (1950), one of which involved quick freezing and vacuum dehydration while the other involved fixation in neutral alcohol-formalin fixative. In addition, radioautographs were prepared of frozen gross serial sections of whole oysters. The latter were particularly useful in studying phosphorus distribution. Their preparation involved quick-freezing the whole oyster, rapidly preparing gross sections three to four millimeters in thickness of the frozen specimen, and clamping them against cold nuclear track plates. The sections were kept frozen throughout the cutting, clamping, and exposure periods. Eastman NTB nuclear track plates with a ten-micron emulsion proved satisfactory, even when exposure involved prolonged storage in a freezer. The simplicity of the process permitted the preparation of radioautographs of serial gross sections of a number of oysters with a minimum of time and equipment. Since the sections remained frozen throughout, there was no loss or movement of soluble radioisotopes.

The oysters used were Delaware Bay market-size oysters. Nearly all the oysters were from a single lot which was stored in trays in the Bay. Small groups were brought to the laboratory by car periodically. There they were kept in a cold room, if not needed immediately. When stored in a cold room, they were placed in sea water at 20° C. for 24 hours before use, and were then transferred to fresh sea water. The responses of stored oysters were normal when compared with freshly collected ones.

All oysters were scrubbed with a stiff brush and washed in running water to remove sand, loose shell fragments, and attached organisms. Measurements of the radioactivity of the outer surface of oyster shells at the end of experiments indicated that there was little adsorption of phosphate ions or accumulation of radioactive phosphorus by such microflora as might have remained attached.

Each of the experiments described represents one of several replications made at somewhat varied initial phosphate concentrations.

Observations

1. Uptake of phosphate by oysters

Groups of six oysters were placed in individual battery jars, each with one liter of water containing 40 micrograms of phosphate phosphorus, to which 20 microcuries of P^{32} as H_3PO_4 had been added. The addition of the radioisotope did not increase the concentration of phosphate ions in the sea water significantly. A constant temperature of 18° C. was maintained. After various time intervals a group of six was removed for assay of the tissues. Duplicate samples were taken of several tissues. All oysters were observed to establish the fact that they were open while in the labelled sea water. The results are shown in Figure 1. The apparent

drop in the mean specific activity of most tissues at 64 hours as compared with 16 hours is not significant. Individual variation was so great that uptake rates are only approximated by this method. This is particularly true in the case of the digestive gland, with a very low and variable uptake rate. It appears, however, that a steady state is reached at about 16 hours.

Several oysters which did not open while in the labelled sea water were also assayed. None showed any radioactivity in the tissues, although in most cases the

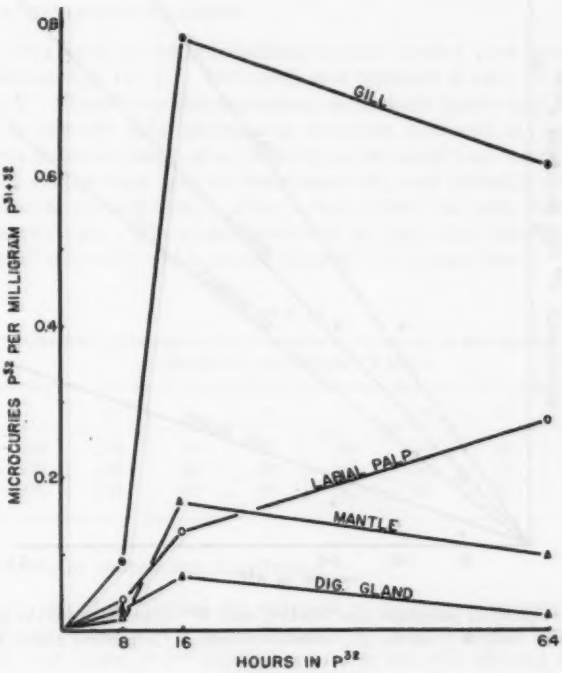

FIGURE 1. Uptake of phosphate ions labelled with P^{32} from sea water by groups of oysters. See text for description.

pallial border was exposed, for the shells had been chipped to facilitate opening them when radioactive.

2. Uptake of phosphate ions by excised oyster tissues

Excised tissues from three oysters were placed in P^{32}-labelled sea water which was aerated and maintained at 18° C. in a water bath. The pieces of tissue were about one cm.^{2} in size. The amount of exposed cut surface varied, being comparatively little in the case of gills and great in the digestive gland and adductor muscle. After time intervals comparable to those in the preceding study, duplicate samples

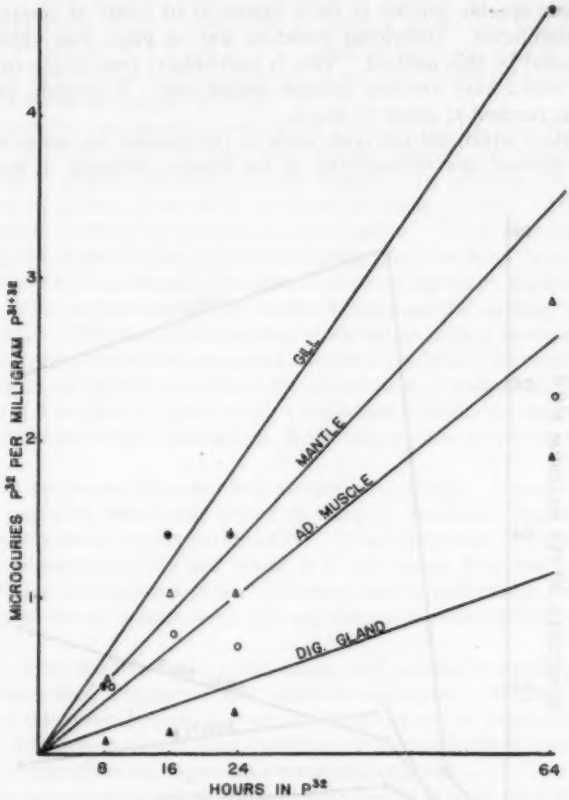

FIGURE 2. Uptake of phosphate ions labelled with P^{32} from sea water by groups of excised tissues of oysters. Gill, ●; mantle, △; adductor muscle, ○; digestive gland, ▲. See text for description.

of each tissue were removed and assayed. Figure 2 shows the results, with computed lines of best fit. The relationship of uptake to time is essentially linear.

3. Labelled phosphorus in the blood

Oysters were placed in labelled sea water as in the first experiment. After various time intervals samples of blood were removed from the heart and assayed. The results are shown in Table I. Each value is the mean of duplicate samples of six oysters. The total blood phosphorus varied in different individuals from eight to 30 micrograms per milliliter. However, there was a labile fraction which was relatively consistent in terms of per cent of total phosphorus.

TABLE I

Mean uptake of phosphate ions by the blood of oysters in sea water labelled with P^{32} at 18° C., expressed as per cent of total blood phosphorus

Hours in P^{32}	1	2	4	8	12
% of blood phosphorus labelled	0.03	0.05	0.08	0.08	0.09

4. Excretion of phosphorus by oysters

Several oysters were placed in individual aerated battery jars containing filtered sea water maintained at 18° C. The water was replaced at two- to four-day intervals for 33 days. Phosphorus determinations were made before and after the water was used. The average daily phosphorus excretion is shown in Table II. Each entry represents the mean daily value for the days the water was in use. The oysters had been in dry cold storage prior to the experiment, and probably voided some accumulated wastes at the beginning. After a week there was little change in the rate of phosphorus excretion. The oysters received no food other than the dissolved or exceedingly small materials which passed through the cotton filter.

TABLE II

Excretion of phosphorus by three oysters, expressed in micrograms of phosphorus excreted per 24 hours

Days:	2	5	7	10	12	16	20	26	33
No. 1	620	100	30	50	40	40	40	20	20
No. 2	560	110	100	80	50	40	40	20	20
No. 3	500	130	100	50	50	40	30	20	20

5. Radioautographs of phosphorus distribution

Oysters were placed in labelled sea water as in the first experiment, after which radioautographs were prepared of several tissues and of gross serial sections of whole oysters. After four hours in P^{32}-labelled sea water the gills showed P^{32} to be generally distributed in them. Other tissues showed no activity, except one specimen which showed activity in one of the peripheral blood vessels of the mantle. After 12 hours in P^{32} most tissues produced radioautographs of nearly uniform density. After 24 hours radioautographs were still uniform though more intensely darkened.

DISCUSSION AND CONCLUSIONS

After periods of one to four hours of exposure to $P^{32}O_4$-labelled sea water, P^{32} is detected in oysters only in the gills and blood taken from the heart, indicating that most, if not all, phosphate ions absorbed directly from sea water enter through the gills and reach other tissues via the blood. After 12 hours only about 0.1 per cent of the total blood phosphorus is derived directly from dissolved phosphate ions.

This small labile fraction may be one of the principal limitations of uptake rate for the organism as a whole. However, additional factors may limit the rate of uptake of phosphate ions by individual tissues. In repeated experiments with excised tissues, their respective uptake rates always fell in the same order. This suggests that each has a characteristic relative uptake rate.

The observation that radioautographs of the tissues of oysters which had been in sea water containing $P^{32}O_4$ ions were uniformly darkened suggests to us that most of the phosphate ions remain in the tissue fluids for the first 24 hours after absorption. At least, there is not enough concentration of the labelled ions in any tissue to cause a noticeably greater darkening of any portion of the radioautographs. The radioautographs of Bevelander (1952), however, show that in some mollusks a fraction is incorporated into insoluble materials within this period. If this is also true in the case of oysters, and probably it is, then the amount of absorbed phosphorus converted to water-insoluble compounds in 24 hours must represent a small fraction of the total absorbed phosphorus.

If in experiment (1) we take 0.1 microcurie per milligram of phosphorus as the approximate mean specific activity of the whole oyster after 24 hours in labelled sea water and assume (on the basis of many analyses) that the average oyster weighs 10 grams and contains 20 milligrams of phosphorus, then the oysters in this experiment absorbed four micrograms of phosphorus in 24 hours. The basal phosphorus loss through excretion is 20 micrograms in 24 hours. The amount of phosphorus obtained from ions in sea water in this case is about 20 per cent of the basic requirement of the oyster. However, in other experiments with different groups of oysters the fraction of the basal requirement obtained from ions in sea water varied from five to 50 per cent. These values must be considered to be only approximations.

The levelling-off of specific activity in the intact tissues, as contrasted with the continued accumulation in excised tissues, suggests that in the former the uptake is balanced by losses from the tissues. The physiological significance of this steady-state cannot be judged from available information.

Leenhardt's (1926) studies of *Gryphaea* (*Crassostrea*) *angulata* indicate that the gill circulation is by-passed by the principal blood vessels, but that there is a small exchange at all times between the gill circulation and the general circulation. If this is also true of *C. virginica*, it may be one factor limiting the utilization of dissolved phosphate. However, the gills, which obtain phosphate ions from the water without mediation by the blood, also reach a steady-state of uptake after about 16 hours. Therefore, some limiting factor other than the rate of circulation and the amount of phosphorus carried by the blood would seem to exist.

Since the gills take up relatively greater amounts of phosphate from the water and obtain it directly, it is interesting to speculate that the gills may be autonomous to some degree. They may obtain a substantial part of their nutritional needs directly by absorption and local phagocytosis.

The observation that no P^{32} entered oysters which remained closed during their exposure to labelled sea water, even when the margins of their shells were imperfect, shows that the pallial curtains are capable of completely sealing off the mantle cavity from the external medium. It further verifies the observation that the tissues of the pallial curtain do not play a large role in the direct absorption of phosphate ions from sea water.

A high degree of physiological variability was noted in the oysters used in this work, although every effort was made to use oysters from a single local population and of one size class for any given experiment. Great variability was also found by Wilbur and Jodrey (1952) in the shell deposition process. Space limitations and the large volumes of radioactive sea water involved made the use of larger groups of oysters impractical.

It is probable that other dissolved materials are absorbed through the pathway which has been demonstrated for phosphate ions. Preliminary studies with Ca^{45} indicate that relatively greater amounts of calcium are absorbed by the gills and carried in the blood. This may be the principal source of calcium for shell deposition.

Summary

1. Phosphate ions in sea water are absorbed by oysters, principally by the gill, and are carried by the blood to all parts of the organism.

2. Directly absorbed phosphate ions are incorporated into the tissues of adult oysters slowly, most remaining in water-soluble forms 24 hours after absorption.

3. Significant amounts of phosphorus may be obtained by oysters through the direct absorption of phosphate ions from sea water.

4. After 12 hours in $P^{32}O_4$, directly absorbed phosphate ions make up less than 0.1 per cent of the total blood phosphorus. This may be one of the several factors which limit the utilization of phosphorus from sea water.

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THE ROLE OF SPECIFIC SURFACE ANTIGENS IN CELL ADHESION. PART I. THE REAGGREGATION OF SPONGE CELLS [1]

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In the "auto-antibody concept" of Tyler (1940, 1942, 1946, 1947) and the "molecular ecology" of Weiss (1941, 1947, 1950) one important part is the hypothesis that contiguous cell surfaces are normally held together, at least partly, by forces like those between antigens and homologous antibodies. The forces are assumed to be associated with specific macromolecules, of at least two stereochemically reciprocal types per cell, so held in the cell surface that they can combine from cell to cell. The hypothesis has been shown to be compatible with a diverse array of observational and experimental data, particularly by Tyler (1947) and Weiss (1947). However, both workers have emphasized the need for new experiments. "It is obviously essential for us first to obtain further evidence for or against the existence of such antigen-antibody like systems of complementary substances within cells" (Tyler, 1947; p. 17). "The subtle means by which cells can recognize each other and their appropriate environments are still wholly conjectural" (Weiss, 1950; p. 184).

It has been proposed (Tyler, 1947) that for further investigations along this line, the phenomena of selective reaggregation of dissociated sponges (Wilson, 1910; Galtsoff, 1925) and of the segregation of cells that occurs in certain combinations of embryonic tissues (Holtfreter, 1939) should constitute favorable material.

One type of experiment is suggested by the proposal that *macromolecules* are involved. Such molecules might act as antigens when injected from one species into another. Antisera so produced should affect, in a predictable way, processes involved in cell adhesion. Experiments of this type are reported here on dissociated *Microciona prolifera* and *Cliona celata*. In a second paper (Spiegel, 1954) experiments on embryonic cells of *Rana pipiens* and *Triton alpestris* are reported.

NORMAL REAGGREGATION AND SEGREGATION

A review of the essential phenomena in sponge reaggregation and segregation may be a useful preliminary to consideration of the experimental results.

Wilson (1907) first showed that a sponge could be dissociated, by pressing through bolting cloth, into isolated cells which would form a number of reaggregates within a day's time. Galtsoff (1925) showed that clusters of about 2,000 or more cells are capable of reorganizing, after 5–6 days under proper conditions, into

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perfect miniature sponges. Various aspects of the phenomena have been studied by Wilson (1910, 1932), Galtsoff (1923, 1925, 1926, 1929), de Laubenfels (1927, 1932) and Brøndsted (1936) among others, and the following account is based partly on their observations and those of the present author.

After disaggregation the cells slowly settle down. Upon coming in contact with the substratum, archeocytes and pinacocytes flatten out, adhere and put forth hyaline pseudopodia. They begin to move almost immediately. The archeocytes are more active and move at about 0.6–3.5 microns per minute, changing direction apparently at random roughly three times per hour. Under extremely favorable conditions their velocity may reach 20 microns per minute (Galtsoff, 1925). Movement continues for about 24 hours.

Upon coming in contact with one another, the cells generally coalesce. The plasma membranes of the apposed cells seem to join together zipper-wise, proceeding from the area of first contact. After 5–10 minutes, the surface of union between the two cells is indistinct or has vanished and it appears that the clear outer hyaloplasm has formed a common matrix for the two inner granuloplasms which remain distinct and separate from one another.

As new contacts continue to occur, larger and larger aggregates are formed, the number and size depending on the density of the original suspension and on temperature, osmotic pressure, ionic composition, pH of the medium, etc. Aggregates increase in size by addition of single cells or by coalescence with other aggregates. Under Galtsoff's standard conditions some aggregates were about 0.5 mm. in diameter, consisting of roughly 2000 cells, after one day. Neither number nor size of aggregates changes further after ameboid movement stops at the end of 24 hours.

Present observations reveal certain additional features of interest. *Movements of granules during cell coalescence.* In Microciona while a pair of cells is coalescing, their granules undergo a series of intense movements and shifts in position, resulting in a striking reorganization of the granuloplasm. The granules which seem originally to be distributed at random in the cytoplasm of an isolated cell reorient so as to lie immediately inside the surface of the granuloplasm. The archeocyte nuclei, which previously had been partly or wholly obscured by the granules, are now quite evident with conspicuous light green nucleoli. *Fusion of hyaloplasm in cell coalescence.* An aggregate of two cells is able to put out a pseudopodium about 15 microns long which is approximately twice that formed by an isolated cell. An aggregate of three cells may form pseudopodia ranging from 30 to 35 microns in length. Large aggregates of 100–200 cells occasionally put out pseudopodia 200–300 microns long, of quite striking appearance. In general pseudopodium size varies with about the 1.2 power of cell number. The hyaloplasm of all the cells evidently acts in some sense as a unit, freely available to the aggregate's single pseudopodium. The microscopic appearance during coalescence suggests that the interface between the hyaloplasms of two coalescing cells disappears.

EFFECTS OF ANTISERA ON REAGGREGATION AND SEGREGATION

Materials and Methods

Specimens of *Microciona prolifera* (the large encrusting red sponge) and *Cliona celata* (the yellow sulfur sponge) were employed. The colonies were kept in running sea water in the experiments done at the Marine Biological Laboratory. For

work at the University of Rochester, living colonies were shipped from Woods Hole by Air Express and kept in sea water, replaced at two-day intervals, at a temperature of 1–2° C. Experimental results were similar under the two maintenance methods.

Quantitative studies of reaggregation. For the experiments, it seemed desirable to have a quantitative expression of the extent of reaggregation at successive times. The following method was used.

For each series of observations a fragment of fresh sponge was carefully dried with filter paper and a one-gram portion squeezed through bolting cloth into 40 ml. of sea water. Ten ml. of the suspension were transferred to a syracuse dish and examined under the microscope at 430 × magnification. At intervals the aggregates were counted in each of five microscope fields taken at random, and the values were averaged. For these counts "aggregate" was defined as any coalesced group of *four or more cells,* and it is in this sense that the word will be used hereafter. Four cells constitute the smallest grouping that can be readily recognized as an aggregate as distinct from cells in contact, in routine examinations.

Typical results over a 24-hour period are shown in Figure 1. Such a figure will be called a reaggregation curve and each of its ordinates, an aggregate count.

The curve is satisfactorily reproducible under standard conditions; as an example, seven unselected curves are shown in Figure 2. Up to four hours the extreme variation of counts at any one time is about two aggregates. Between four and eight hours counts are somewhat more variable. But they are quite uniform again at 24 hours, at least under normal conditions: 12 such counts, unselected, range from 3.9 to 4.5. The progress of reaggregation as illustrated by these curves

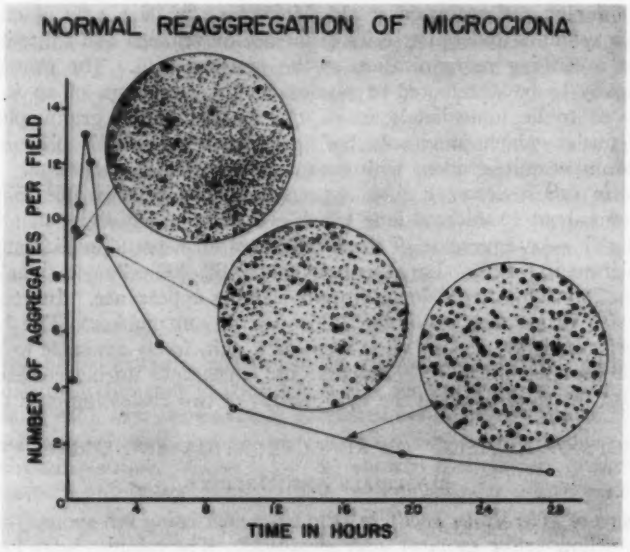


FIGURE 1. Reaggregation of *Microciona prolifera* in sea water.

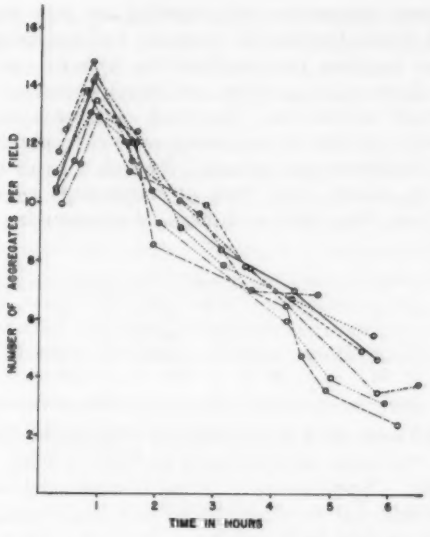


FIGURE 2. Reaggregation of *Microciona prolifera* in normal serum; seven unselected curves demonstrating reproducibility under standard conditions. Essentially the same curve is obtained in sea water.

shows an initial increase in number of aggregates (of small size) to a maximum at about one hour, followed by a decrease in number (fusion of aggregates with corresponding increase in size).

In a mixed suspension of the red-colored Microciona and the yellow Cliona cells, 24 hours after dispersal, the aggregates are observed to be species-specific, as reported by Wilson (1910), Galtsoff (1925) and de Laubenfels (1927). A few cells of one type in an aggregate prevailingly of the other species cannot readily be distinguished. If such admixture occurs, its extent is too slight to be readily detectable.

The Microciona aggregates formed in a mixed suspension are more numerous and correspondingly smaller than in a pure Microciona suspension. They are, however, normally well rounded with a well-defined hyaline membrane. Similarly, the Cliona aggregates, more and smaller than in pure Cliona preparations, have the characteristically jagged contour but easily seen hyaline coat of pure Cliona suspensions. Occasionally an aggregate of Microciona and one of Cliona are observed in close contact, but their hyaloplasms remain separate and the two fail to adhere.

It is important, for interpreting the experimental results, to note here that the condition seen at 24 hours is preceded by an initial temporary intermixture of the two types of cells. But at 4–8 hours, after dissociation, in most aggregates islands of cells of one species are seen in a matrix of the other species; they are apparently separating from it.

The smaller size of aggregates in mixed suspensions is perhaps partly a consequence of the sorting out: contacts which would lead to a permanent increase of

aggregate size in a pure suspension are cancelled out in a mixed preparation by the later sorting out where interspecific contacts had occurred. To some extent each species may also interfere just mechanically with the other's aggregation as Galtsoff (1925) has shown glass particles and starch grains to do.

Preparation of stock suspensions. For each of these a piece of fresh sponge was cut into fragments 2–3 mm. across, which were carefully dried on filter paper. A known weight of fragments was squeezed through a small bag made of bolting silk, mesh 20, into sea water. Six types of suspensions were used, and freshly prepared before each use; they were as follows, M representing Microciona, and C, Cliona:

Stock suspension	1	2	3	4	5	6
Sponge (g.)	6.2M	6.2C	6.2M 6.2C	1M	6.2C	1 M 6.2C
Sea water (ml.)	20	20	40	40	40	40

Suspensions 1, 2 and 3 were used in preparations of antisera; their composition was so chosen as to give the same total nitrogen per ml.; 1.3 mg. in each, by micro-Kjeldahl determination. Suspensions 4, 5 and 6 were used in tests of antiserum effects; their composition was so chosen as to give about equal initial numbers of free cells per microscope field in 4 and 5.

Preparation of antisera. For most of the experiments the antisera were derived from nine New Zealand giant rabbits, three for each suspension. Nine subcutaneous injections of 0.1 ml. of stock suspension, were given over a period of three weeks.

At 8 days after the last injection blood was collected in sterile 50-ml. Pyrex centrifuge tubes, allowed to clot at room temperature, kept at 40° C. for one hour, the clot loosened, and the blood centrifuged at 350 G for 15 minutes. The clear straw-colored serum was pipetted off and the following were added per 50 ml. serum: 0.2 mg. Penicillin G potassium, 0.2 mg. Streptomycin calcium chloride complex and 0.2 mg. Aureomycin. The mixture was kept in sterile serum bottles at 1° C. until use.

Serum obtained from animals injected with Microciona cells will be referred to hereafter as anti-Microciona serum; others will be similarly called anti-Cliona and anti-Microciona: Cliona.

Test of antiserum titers. These were done to determine, by standard methods, that the sera actually contained antibodies to sponge antigens. A 6.2-gram portion of Microciona was homogenized for five minutes in a Ten Broeck glass homogenizer with 20 ml. of 0.01 *M* phosphate buffer at pH 7.2 and containing 0.85% NaCl. The homogenate was centrifuged at 485 G for 20 minutes and the supernatant collected. The same procedure was carried out with 6.2 grams of Cliona cells in 20 ml. of buffered saline and with 6.2 grams Microciona plus 6.2 grams Cliona in 40 ml. of buffered saline.

Precipitation tests were set up by layering 0.5 ml. of undiluted extract and of five 10-fold dilutions (1:10 to 1:100,000 in buffered saline) over 0.5 ml. of homologous, undiluted antiserum, or over saline or normal serum as controls in 10 × 75 mm. serological tubes. All tubes were incubated at 37° C. for one hour and then

examined for presence of a precipitate ring at the interface. All nine sera produced rings of antigen-antibody complex at all antigen dilutions down through 1:10,000. Controls were negative. The tests show, then, the presence of antibodies to sponge antigens extractible in saline, although these do not necessarily mean that cell surface antigens are involved.

Tests of antiserum effects on reaggregation. Three ml. of an antiserum were mixed with an equal volume of double-strength van't Hoff solution alpha (58.44 grams NaCl, 1.62 grams KCl, 2.24 grams $CaCl_2$, 16.18 grams $MgCl_2.6H_2O$, 9.38 grams $MgSO_4.7H_2O$, distilled water to one liter mark), producing a medium approximately isosmotic with sea water. Four ml. of suspension 4, 5 or 6, freshly prepared, were added. Controls were 4-ml. portions of the same cell suspension added to three ml. of double-strength van't Hoff solution plus three ml. of serum from a normal uninjected rabbit (18 experiments), or to six ml. of ordinary strength van't Hoff (four experiments). In five experiments there were no simultaneous controls.

Altogether 63 antiserum and 22 control preparations were observed in 27 experiments; normal reaggregation was also followed in 13 other preparations, 10 in normal serum and three in van't Hoff solution.

Results

Microciona in anti-Microciona serum

Reaggregation curves for Microciona cells during the first three hours in normal serum and in two anti-Microciona sera are shown in Figure 3 with photomicrographs at 2.75 hours. In two other antiserum preparations studied by reaggregation curves, results were obtained scarcely distinguishable from the two pictured here.

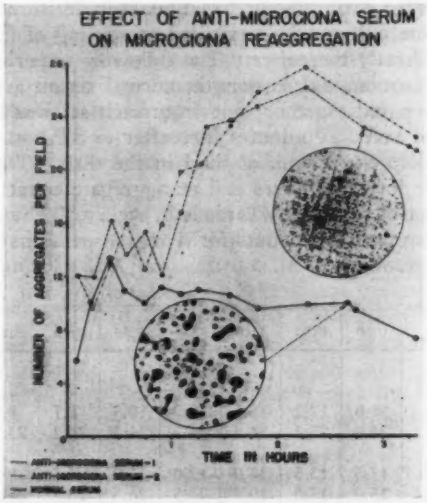


Figure 3. Effect of anti-Microciona serum on Microciona reaggregation.

The curve in normal serum is slightly different from that usually obtained in sea water or normal serum: its peak is at 0.5 rather than one hour, and it is lower by about two aggregates from 0.75 to 2 hours. Seven other curves in normal serum (Fig. 2) are indistinguishable from the results in sea water.

Small but significant differences between normal serum and antiserum appeared within one hour. The peak counts in antiserum, 26 and 27, occurred at 2.25 hours when the count is always close to 10 in normal serum; peak values in the two other antisera were 27 and 28 at 1.75 hours. From then on the count in antiserum does not seem to change significantly: 25 counts at 24 hours ranged from 21 to 32 with an average of 28.6. The apparent trend downward in Figure 3 from 2.25 to 3.25 hours is probably not significant: the drop is well within the range of variation at 24 hours, and there was no such decrease in the two other reaggregate count dishes. So there is probably no reaggregation in antiserum after about two hours.

In normal serum at 2.75 hours, less than 10% of the cells remained dissociated; the rest were included in aggregates of roughly 100–2000 cells (Fig. 3). In antiserum about 90% of the cells were still dissociated at that time, and most of the aggregates contained fewer than 20 cells. A few, however, consisted of 100 or more cells.

There were two other striking differences at 2.75 hours. In normal serum the aggregates, seen from above, were circular, oval or—where two were fusing—dumbbell shaped. Their borders consisted of small and about equal portions of curved cell surfaces. In antiserum even the largest aggregates had less symmetrical shapes and much more irregular borders. The second difference was that in antiserum a large proportion of the cells were in loose clusters; adjacent cells were scarcely more than tangent, with very little surface in contact.

Altogether, the differences at 2.75 hours show that much less reaggregation occurs in antiserum than in normal serum even before two hours. The striking increase in aggregate count during the first two hours in antiserum evidently does not represent reaggregation. It can be attributed to settling of aggregates present at the start or formed shortly thereafter. The following experiment illustrates this.

Ten standard Microciona suspensions in normal serum and 10 in anti-Microciona serum were prepared together; one from each set was killed at 30 minutes after dissociation, and every 20 minutes thereafter to 3.5 hours, by adding 10 ml. of 95% ethyl alcohol to the 10 ml. of fluid in the dish. The killed suspensions were allowed to settle for three hours and an aggregate count (as usual, the average of five fields chosen at random) was made in each. The results were as follows, with the corresponding ordinary counts for living suspensions given in lines 3 and 4 below.

Time (hours)	0:30	0:50	1:10	1:30	1:50	2:10	2:30	2:50	3:10	3:30
Aggregate counts										
Killed suspensions										
1. Normal serum	11.0	12.2	12.1	10.0	10.3	8.7	8.8	7.6	5.1	4.6
2. Antiserum	24.0	25.2	24.8	24.7	24.7	24.2	23.9	25.0	25.1	24.4
Living suspensions										
3. Normal serum	11.5	13.5	13.0	12.0	11.0	9.5	9.0	9.0	8.0	7.5
4. Antiserum	15.0	15.0	19.5	23.5	26.5	26.5	26.5	25.5	25.0	—

Line 3 is the average of the seven curves in normal serum; line 4, of the four in antiserum. Comparison of line 2 with line 4 shows that the increase in aggregate count recorded for the living suspension in antiserum (line 4) is very likely due to the settling of aggregates already present at the start.

Assuming that there are on the average about 25 aggregates (of small size) present at the start, the value of 11 or 12 for the normal serum at 30 minutes (lines 1 and 3 above) means that reaggregation occurs much more rapidly within this initial period than later on. This is to be expected since it is reasonable to assume that the rate of reaggregation will depend on the number of free units (single cells and aggregates) present, and this number decreases with time.

This analysis assumes, then, that dissociation does not yield simply a suspension of single cells but that many small clumps of cells pass through the bolting cloth. In fact observations made as soon as possible after dissociation (5–10 minutes) show approximately half of the cells to be in aggregates ranging in size from four to 10 cells with occasional clumps of 40 to 50 cells. This has been noted by previous investigators (Wilson and Penney, 1930; de Laubenfels, 1932).

Wilson and Penney (1930), describing an expressate, refer to "compact groups of collar cells" and "pieces of chamber walls," and show a group of 12 cells in their Figure 20. They do not indicate what proportion of the freshly-pressed out cells are in such groups. De Laubenfels (1932) points out that the meshes of bolting cloth are (p. 49) ". . . several times the diameter of a sponge cell, often as much as 200 microns in diameter of opening. If the mesh acted as a sieve, it would still be possible for groups of dozens of cells to come through. As a matter of fact, entire flagellate chambers do come through the cloth as aggregations of about 25 microns to 30 microns in diameter. . . ." He does not say how frequent such groups are.

Altogether, there is little reason to conclude that any reaggregation of Microciona cells occurred in anti-Microciona serum, at any time. In fact the following observations indicate that the aggregates present from the start tended to shed cells: (1) At the initial count of a reaggregation curve 50% of the cells were noted to be in aggregates as compared with many more free cells at 1.5 hours, and as compared with the upper photomicrograph of Figure 3 in which, before reduction, it was estimated that no more than 20% of the cells are in aggregates. (2) The number of aggregates as large as 40–60 cells, which was low at the start, decreased with time.

The failure of reaggregation, and the possible further disaggregation were not due to death of the cells. A suspension of Microciona in antiserum was centrifuged after 12 hours and decanted. The precipitated cells were re-suspended in sea water and swirled for about five minutes. The process was repeated and the twice-washed suspension was set aside in a syracuse dish. Nineteen hours later the cells were found to have reaggregated normally. A second experiment, with only one washing, gave identical results.

The above experiments show that what can be seen at the bottom of a dish of dissociated sponge "cells" during the first few hours may depend on several processes, in a rather complex way:

1. Passage of cell groups through bolting cloth;

2. coalescence among these groups while the suspension is being prepared for observation;

3. contact of some "cells" with the substratum, and adhesion, during preliminary swirling;

4. settling of initially suspended "cells" throughout the first 2 hours;

5. contact and coalescence among cells and aggregates creeping on the substratum.

A reaggregation curve during the first few hours reflects these processes jointly. But 1, 3, and 4 are really irrelevant to the phenomena of primary interest here. Furthermore the Microciona experiments in anti-Microciona serum showed that processes 2 and 5 operate alike, so that no significant information is obtained within the first few hours that could not be more easily gotten from any single later observation. Since the same would presumably be true for Cliona and for other antisera, attention was confined in subsequent experiments to observations at 24 hours after dissociation (when cell movement ceases and no further coalescence occurs).

Other combinations of sponge and antiserum

Microciona (*Fig. 4*). In anti-Cliona serum there were no signs that reaggregation had been inhibited. The aggregates in anti-Cliona serum were as large or larger than normal (Fig. 4). Five experiments gave consistent results, the aggregates counts at 24 hours averaging about 4.3.

In anti-Microciona:Cliona serum, Microciona formed aggregates of number and size intermediate between those in normal serum and in anti-Microciona serum; reaggregation was partly inhibited.

Cliona. There were more, and smaller, aggregates in anti-Cliona serum than in normal serum, indicating an inhibition of reaggregation analogous to that of Microciona in anti-Microciona serum.

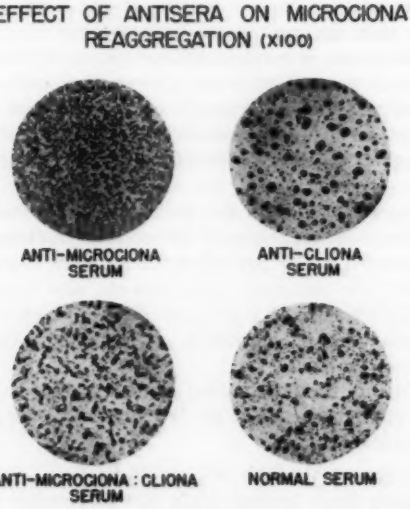


FIGURE 4. Reaggregation of Microciona in antisera.

In anti-Microciona:Cliona serum, there were fewer aggregates than in anti-Cliona serum but more, and smaller, than in normal serum. The inhibition was partial like that of Microciona in the same serum.

With anti-Microciona serum, there were fewer and larger aggregates than in anti-Cliona serum but smaller than in normal serum, and scarcely larger than in anti-Microciona:Cliona serum.

Microciona-Cliona mixtures. In normal serum the initially mixed cells sorted out. The Microciona aggregates were smaller than are formed by pure Microciona as were also the Cliona aggregates. The probable reason for this has already been suggested above and has nothing to do with their surface antigens.

In anti-Cliona, as in normal, serum, there were no mixed aggregates and the Microciona aggregates were normal, though smaller than formed by Microciona alone in normal or in anti-Cliona serum. The Cliona components of the suspension were much like Cliona in anti-Cliona. The results are what would be expected if anti-Cliona serum contained antibodies to surface antigens of Cliona but not Microciona.

In anti-Microciona serum, the mixed suspension did not form any large aggregates of the Microciona type, but many isolated cells and small clusters were seen. The larger aggregates were found to consist of Cliona cells. Most of them were about as large as formed by Cliona alone in anti-Microciona serum. They were smaller, though, than those of Cliona alone in normal serum. This can be interpreted as a consequence of the cancelling of previous associations by ejections of Microciona cells from mixed clusters as suggested above for mixed cells in normal serum.

In anti-Microciona:Cliona serum, a result was obtained diametrically opposite to the effect of the other antisera on pure or mixed suspensions. There were large aggregates of general shape intermediate between Microciona in normal serum and Cliona in normal serum. Many of the aggregates were much larger than in any other cell-serum combination. At the time of the experiment it was noted that (1) cells, or cell groups, of the 2 species were intermingled apparently at random throughout the aggregates: at least there were no patches of red Microciona cells or of colorless Cliona cells, but the aggregates were of a uniform, intermediate color; (2) the aggregates had a somewhat porous structure; in the reticulum between pores, the cell-to-cell contacts seemed to be about as close as in either Cliona or Microciona in normal serum.

EFFECT OF CALCIUM ON REAGGREGATION

Galtsoff (1925) and de Laubenfels (1932) have reported that reaggregation is impeded in media outside a limited range of calcium concentrations. But Agrell (1951) was unable to maintain *Halichondria panicea* suspensions as isolated cells in citrate or oxalate solutions. In view of this inconsistency, and considering the apparent role of surface antigens, it seemed desirable to check some of the previous work on the action of calcium. Three types of experiments were carried out: in calcium-free media, in the presence of a calcium-complexing agent, and in high calcium solutions.

Effects of calcium-free media. Galtsoff (1925) reported that in NaCl or KCl solutions, the cells do not move and no coalescence occurs, and that even after 24 hours, the substratum is covered with single cells which die about a day later.

Following Galtsoff's methods a piece of Microciona weighing two grams was squeezed through bolting cloth into 20 ml. of 0.52 *M* NaCl (isotonic). The suspension was centrifuged at 62 G for five minutes and the supernatant drawn off with a pipette; fresh solution was added and the tube containing the cells vigorously shaken. The centrifuging and subsequent washing were repeated three times. Two ml. of this suspension were added to (1) eight ml. of 0.52 *M* NaCl, to (2) eight ml. of 0.52 *M* KCl, and to (3) eight ml. of normal sea water, in syracuse dishes. After 24 hours, the number of aggregates, per microscope field, was counted for five random fields.

Medium	1	2	3
Average no. of aggregates per field	0	0	4.2

At 24 hours, the NaCl and KCl cell suspensions were swirled and large macroscopic aggregates were formed. Identical results were obtained if the cells were suspended for 24 hours in an isotonic NaCl solution and then washed with subsequent centrifuging, three times, re-suspending each time by vigorous shaking. It is clear, therefore, that the cells fail to reaggregate not because they were dead or lacked adhesiveness after 24 hours, but because they had lost the ability to move.

Effects of Versene on reaggregation. It might be argued the NaCl or KCl solutions do not completely deprive the cells of calcium, and in order to check the above observations, it was desirous to find some means of effectively binding calcium specifically, so that it would no longer be available to the cell. Sodium oxalate or sodium citrate can combine with calcium to form an insoluble precipitate but this might disrupt the internal organization of cells and consequently bring about their death. It was necessary, therefore, to use a substance which is capable of forming a strong soluble complex with calcium and which is not toxic to living organisms. Versene (ethylenediamine tetra acetic acid) is such a compound; at pH 7.0 it sequesters calcium from solutions in a 1:1 molar ratio, by chelation.

Two grams of Microciona were squeezed through bolting cloth into 20 ml. of 0.05 *M* di-sodium versenate in 0.50 *M* NaCl, 0.01 *M* phosphate buffered at pH 7.00. The suspension was centrifuged at 62 G for five minutes and the supernatant removed with a pipette; fresh solution was added and the tube containing the cells vigorously shaken. The centrifuging with subsequent washing was repeated three times. It was calculated that all of the calcium and possibly some magnesium ions should at that point have been chelated with Versene. Two ml. of the suspension were then added to (1) eight ml. of the di-sodium versenate solution and to (2) eight ml. of normal sea water, in syracuse dishes. After 24 hours the dishes were examined under the microscope and the aggregates counted.

The same results as in NaCl and KCl were obtained.

Medium	1	2
Average no. of aggregates per field	0	4.5

At this time the bottom of the versenate dish was covered with single isolated cells which did not exhibit either ameboid movement or coalescence. When the dish was swirled large macroscopic aggregates were formed. Identical results were obtained with cells first suspended for 24 hours in di-sodium versenate and then centrifuged followed by washing three times, re-suspending each time by vigorous shaking. Since a normal aggregate count was obtained with cells trans-

ferred to sea water, it is readily apparent that the cells remained alive in the versenate solution.

The above experiments offer additional evidence to support the conclusion that calcium deficiency prevents reaggregation by the inhibition of ameboid movement and does not affect or alter the adhesiveness of the cells to each other.

Effects of calcium excess on reaggregation. Although no role of calcium in cell adhesion is indicated by the effects of calcium deficiency, one might be detected by the effects of calcium excess. Therefore, one gram of Microciona was dissociated into 40 ml. of normal sea water and two ml. of the suspension were added to eight ml. of normal sea water, and to seven ml. normal sea water plus one ml. of 0.35 *M* $CaCl_2$ in syracuse dishes.

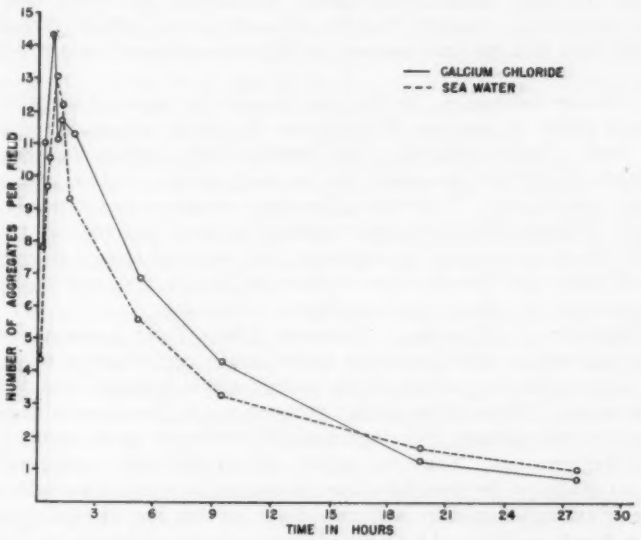


FIGURE 5. Reaggregation of Microciona in normal and high calcium media.

Aggregate counts were made at varying intervals; the reaggregation curves are shown in Figure 5. Differences are well within the limits of variation among preparations in sea water.

In experiments under identical conditions, Galtsoff (1925) found 7.1 times as many aggregates (meaning less reaggregation) in excess-calcium solution as in normal sea water at 24 hours. The difference in results is difficult to explain; it leaves considerable doubt whether reaggregation is significantly affected by increasing the calcium of the medium 4.1 times.

DISCUSSION

The present results show that reaggregation is inhibited by the presence of homologous antibodies. The usual expectation, when a specific antiserum is added to a suspension of the homologous cells, is that an agglutination of the cells will

occur. The first point to be discussed, then, is why such agglutination by antibodies does not appear in the present experiments. There are at least three conditions under which agglutination may not occur even though specific reaction takes place between surface antigens and homologous antibodies:

(1) *Extreme antibody excess.* Under this condition the cells are, presumably, completely saturated with agglutinin before cell-to-cell contacts are made. The cells are incapable of reacting with each other since all their receptor sites are occupied. Such a hypothesis has been offered by McKerns and Denstedt (1950) to account for the invariable persistence of unagglutinated red blood cells in agglutinated samples even when potent isoagglutinating serum is used. Failure of agglutination in antibody excess is, of course, the expectation from the Marrack-Heidelberger-Pauling "mutual multivalence" hypothesis (see Boyd, 1947 for ref.). It does not seem likely, however, that the sera used in the present investigation are of such high titer that the vast majority of cells are saturated before cell collisions take place.

(2) *Univalent antibodies.* In this case it would be assumed that the antibodies formed by a rabbit in response to injections of sponge antigen are chiefly of the univalent type. These antibodies could combine with surface antigens but since each molecule of antibody possesses only a single group reactive with antigen no agglutination takes place. This type of antibody seems to be commonly formed in Rh antisera (Wiener, 1944) and other immune antisera (see refs. in Tyler, 1945). Such a hypothesis is adequate for explaining inhibition of sponge reaggregation by homologous antiserum but offers no explanation for the apparent agglutination of mixed suspensions of cells by the homologous antiserum.

(3) *Structure of cell surface.* Professor Albert Tyler (personal communication) has suggested a third condition under which agglutination may not occur. One may assume that the surface of the sponge cell is a folded, and probably also flexible, structure. Most of the surface antigens would then be so situated that the two or more valence groups of a single antibody molecule would tend to react with antigen on the same cell. Since the surface of a sponge cell is observed to change its shape, as evidenced by pseudopodium formation, it seems reasonable to assume that there is enough flexibility and movement so that the valence groups of the antibody molecule would be able to contact two or more of the receptor sites on the same cell surface. Very few, if any, valence groups would then be available for reaction with receptor sites on other cells. This, then, can account for absence of agglutination by homologous antiserum and the inhibition of reaggregation. It can also account for the clumping obtained with mixed cells (of the two species of sponge) in antiserum vs. mixed cells. Here it is assumed that heterovalent antibodies are formed. Considering (for simplicity) only two valences on the antibody molecule, one may be termed anti-M, the other anti-C. Such antibody molecules would react at one end with cells of one species of the mixed suspension (say M cells) leaving a valence group (anti-C) free to contact the cells of the other species.

The failure of ostensibly multivalent antibodies to cause agglutination has been noted in several immunological systems. Gleeson-White *et al.* (1950) and Coombs *et al.* (1951) have studied the reactions of ox red cells in rabbit or guinea-pig anti-ox sera. The red cells of most oxen fail to agglutinate in such sera. Coombs *et al.* suggest that the failure is due to a deep location of the antigens so that a single

antibody molecule, even if multivalent, after combining with one cell cannot reach receptors of a second cell.

Coombs *et al.* have been able to accomplish the agglutination by certain devices which, in effect, can be considered to supply links between the antibody molecules attached to the different cells. For example, anti-rabbit globulin serum (produced in goats) will agglutinate the ox cells that have been treated with the rabbit anti-ox cells antiserum. This is particularly effective when the procedure is a serial addition of anti-globulin, then globulin, then anti-globulin, which is pictured as forming a longer chain, and provides strong support for the hypothesis of "deeply located" antigens.

This hypothesis does not, however, enable us to account for the agglutination of mixed cells by anti-mixed cells serum. It was therefore modified as described above in terms of interaction of a single antibody molecule with different parts of the same cell surface. Apart from the validity of the interpretation it appears clear from the work of Coombs *et al.* that there can be failure of agglutination, even in presence of multivalent antibody, with certain types of cells.

The effects of antisera on sponge reaggregation in relation to the Tyler-Weiss hypothesis will be considered in a stepwise discussion of the results.

1. *Anti-Microciona serum inhibits reaggregation of dissociated Microciona.* This conclusion can scarcely be doubted from the joint evidence of reaggregation curves up to three hours in standard and alcohol-killed suspensions, aggregate counts at 24 hours, microscopic appearance and size of aggregates at 2.75 and 24 hours, in normal serum and antiserum.

2. *The primary inhibiting effect of antiserum is on the process of adhesion.* The initial step in reaggregation, ameboid movement producing random contacts between cells, is normal in antiserum. But even when cells make contact in antiserum they fail to coalesce. There is no implication here of the mechanism by which the area of contact increases such as was suggested by Schmitt (1941).

3. *Some substance or class of substances "in" the cell surface is essential to the process of adhesion.* This conclusion is based on three considerations. (i) The zipper action, by which two impinging cells increase their area of contact, leads into the persisting contacts between cell surfaces within an aggregate. (ii) It seems probable that the same forces are responsible for the initial adhesive and the permanent contacts. The probability of this proposition is difficult to estimate for sponges because the boundary which can be seen, while two cells are joining, disappears within a few minutes. It is this change to which Galtsoff (1925) applied the term coalescence. Apparently the two hyaloplasms merge and the factors responsible for binding the two cells together then become simply part of the structure of the hyaloplasm. (iii) The *intrinsic* factors that hold cells together are commonly thought to be in the cell surface, *e.g.*, lipids. For many types of cells, extrinsic factors have been shown to be equally important, *e.g.*, calcium in salt linkage between lipids or proteins of adjacent cell surfaces, or "intercellular cement" which may involve a similar chemical bounding of an extrinsic substance to surface materials of two cells (Chambers, 1940). Other possible extrinsic factors are materials such as histones (Schmitt, 1941).

4. From steps 2 and 3, *the primary effect of antiserum is on some substance or substances in the cell surface.* Adhesions of two cells must involve interactions of their surface. It seems extremely unlikely that the antibodies affecting adhesion

would enter the cell producing some change inside which is later manifested in altered properties of the surface. A direct action of antibodies on the surface is more reasonable and can explain the results.

5. From the steps 1 and 4 it is reasonably probable that *anti-Microciona serum blocks the normal action of the materials in the cell surface that are essential for adhesion.*

6. *The only substances in anti-Microciona serum that are not also present in normal serum are antibodies to substances of Microciona that can act as antigens.* This has a three-fold basis. (i) Three anti-Microciona sera gave positive precipitin tests with Microciona extracts; two other anti-Microciona sera had the same effects on reaggregation as the three with precipitin tests. Therefore all five almost certainly contained antibodies. (ii) Three normal sera gave negative precipitin tests with Microciona extracts; two others, not precipitin tested, had no effect on reaggregation nor did the three tested. Therefore all five almost certainly had no antibodies to Microciona. (iii) Antisera do not normally differ qualitatively from normal sera, where comparable animals are used, except by presence of antibodies (Boyd, 1947).

7. From steps 5 and 6, *the normal role of certain surface antigens in adhesion is blocked by homologous antibodies present in antisera to Microciona cells.* The antigens will be represented by the symbol M, and the antibodies by anti*M*.

8. From comparable, less extensive tests, and by similar reasoning, *antigens A, in the surface of Cliona and essential in adhesion, are blocked in function by antiA in anti-Cliona serum.*

9. *M and A are specific antigens.* The main evidence here is that anti-Cliona serum does not block reaggregation of Microciona; the reasoning is analogous to steps 1–7. With Cliona in anti-Microciona serum there is some inhibition of reaggregation. One possible explanation for this is that Microciona possesses below the cell surface an antigen immunologically similar to a surface antigen of Cliona.

10. *M is inactive with respect to adhesion because it is combined with antiM.* This follows from step 7 and from the general knowledge that the only significant action of antibodies, under circumstances analogous to the present, is to combine with their homologous antigens (Boyd, 1947). The compound will be represented as M-anti*M*, without any implication as to the actual number of molecules of either M or anti*M* in the compound.

11. *M is not completely removed from a blocked cell.* This must be concluded from the fact that reaggregation can occur after washing an antiserum-treated suspension in sea water. It does not seem likely that such washing dissociates an antigen-antibody complex since such dissociation evidently involves more drastic treatment, such as high salinity (Heidelberger and Kabat, 1937) or high pH (Liu and Wu, 1938; Sternberger and Pressman, 1950). A more likely interpretation is that M extends below the surface; perhaps the entire hyaline layer is a gel of M and that M, at the surface, normally goes slowly into solution in sea water and is continuously replaced by M manufactured below. On this basis then, M-anti*M* would leave the surface slowly, or more rapidly by washing the cells, which would then be capable of reaggregating.

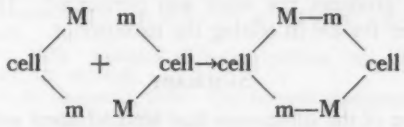
12. *The region of the M molecule to which antiM attaches is either* (a) *the site that is involved in adhesion, or* (b) *near enough to that site so that antiM overhangs or somehow interferes with the activity of the adhesion site.*

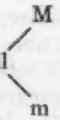
This is based on step 11 and 3 other considerations. (i) There seem to be only two possible ways of operation of M in normal adhesion: either through an extrinsic binder like calcium, *e.g.*, cell-M-Ca-M-cell; or by some type of direct bonding between M of one cell and M or some other substance m of another cell, *e.g.*, cell-M-m-cell, as envisaged by Tyler and Weiss. In this case a single cell can be assumed to have both M and m on the surface. (ii) The former seems unlikely considering particularly the failure of Versene to prevent reaggregation and the apparent absence in Microciona of anything able to act as an intercellular cement. De Laubenfels (1932) cites some evidence suggesting that, in dissociated *Iotrochota birotulata*, an intercellular slime is dispersed throughout the medium and brings cells or cell groups together, presumably by some sort of contraction. The writer has not seen, or had evidence of, any such material in Microciona. When a suspension is stirred the cells or cell groups move independently of each other; there are no signs of intercellular traction, nor any visible strands that could be identified as slime. (iii) Conclusion 12 follows from the remaining possibility: that the process of adhesion is essentially cell-M + m-cell → cell-M-m-cell.

13. *Alternatives a and b of step 12 cannot be readily distinguished.* The antibodies might well be directed against some constituent of the surface that is not directly concerned in reaggregation. Such bound antibodies might partly overlie the chemical sites involved in adhesion. This possibility cannot be distinguished on the basis of present evidence from the possibility that the antibodies combine directly with the chemical sites involved in adhesion.

14. It is likely that *adhesion depends on the presence, in the cell membranes, of two intra-molecular configurations with the reciprocal structural relationship of antigen and antibody.* It is possible that the reciprocals exist either within a single molecular species or in distinct substances. For the reactant reciprocal to M, there will be retained the symbol m introduced at the end of step 12 without implying that these sites are identical with the antigenic sites against which the antibodies are directed. Adhesion implies that M and m are each present in the cell surface in such position and arrangement that M and m of one cell can, under proper circumstances, combine with m and M of another cell. If this is so, within the surface of any one cell M and m must be so held in position that they cannot combine with each other.

The structure of a cell surface, with respect to adhesion, would then be crudely analogous to a balloon on which many snap-fastener parts have been attached, male and female separately but interspersed. And the *unit* reaction in adhesions would be



The M-m bonding would be that of antigen to antibody; the  bonding would be whatever holds the components of a cell surface in position.

Is this necessarily a correct picture? Obviously a yes-or-no answer can not be given. At best one might hope to calculate a probability that it is: one would have to estimate the probability of correctness at each step in reaching the final conclusion, and multiply those component probabilities together. Here too, it seems impossible to give a specific answer.

One conclusion of some immunological interest seems inescapable; that, when Microciona and Cliona cells are injected together into a rabbit, some antibody molecules are produced with one or more groups reactive to M or m *and* one or more groups reactive to A or a. These may be termed mixed or heterovalent antibodies. If such antibodies are formed—presumably along with homovalent anti*M*, anti*m*, anti*A*, and anti*a*—two opposing processes will together determine what happens when a mixture of Microciona and Cliona cells is put into anti-Microciona: Cliona serum:

1. blockage of M, m, A, and a, by the homovalent antibodies;

2. linkage of heterovalent antibodies to M or m of a Microciona cell *and* to A or a of a Cliona cell.

Thus of the total antibody combined with M or m, a certain proportion is heterovalent, with a second group that can react with the remaining uncombined A or a. This should lead to aggregation between cells of the two species as described in the fourth paragraph of the discussion.

Examination of standard texts and monographs does not give the non-immunologist any clear impression whether or not it is reasonable to account for the present results as has been done, in terms of heterovalent antibodies. Burnet and Fenner (1950) say (p. 36) "There is fairly conclusive evidence that an antibody cannot function as such against two different antigens," referring to work of Dean, Taylor and Adair (1935).

Roesel (1951), on the other hand, has obtained evidence indicating that heterovalent antibodies are produced in the rabbit in response to injections of a mixture of the bacteriophages T_2 and T_5. Race, Sanger, and Lawler (1948) believe that the majority of anti-C (anti-rh') sera are really anti-C + C^v in specificity since either antigen, C^v or C, is capable of removing both antibodies. They feel that both components, anti-C and anti-C^v are on the same molecule.

There is therefore some evidence in favor of heterovalent antibodies and the assumption of such antibodies offers a satisfactory explanation of the experimental results obtained with mixed cells in mixed antiserum.

The author wishes to express his deep appreciation to Professor Donald R. Charles under whose guidance the work was performed. He is also indebted to Professor Albert Tyler for aid in editing the manuscript.

Summary

1. An investigation of the substances that bind adjacent cell surfaces together is reported here, using two species of sponge, *Microciona prolifera* and *Cliona celata*. Antisera were made, in rabbits, to cell suspensions of each species and to a mixture of cells of both species.

2. Reaggregation of dissociated cells was reversibly inhibited in the homologous antiserum. In normal serum containing cells of both species, the cells sort out to

form aggregates consisting entirely of either one species or of the other, *never* of both species. In an antiserum vs. both species, large aggregates were observed which consisted of cells of both species distributed at random throughout each aggregate.

3. Calcium-free and high calcium media had no effect on reaggregation.

4. The results are compatible with the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces like those between antigens and homologous antibodies.

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THE ROLE OF SPECIFIC SURFACE ANTIGENS IN CELL ADHESION. PART II. STUDIES ON EMBRYONIC AMPHIBIAN CELLS [1]

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Holtfreter (1943) first demonstrated that embryonic amphibian cells could be disaggregated by exposure to his standard solution (Holtfreter, 1931) at high pH. Townes (unpubl.) has utilized this technique to investigate the problem of cell affinities. The following account is based on these previous investigations.

If an amphibian gastrula or neurula is exposed to Holtfreter's solution at a pH greater than 9.6, the pigmented surface coat becomes soft and is partially dissolved. "Within a few minutes cracks appear in it; new disruptions follow, until the whole surface is broken up into small dark islets, surrounded by much larger white interfaces. A closer examination reveals that each islet is the centre of a group of cells, attached to it in a radial orientation" (Holtfreter, 1943; p. 292).

Under the action of the alkali, the surface coat, which is disrupted at the cell boundaries, retracts in the form of a black cap to the pole of each cell. This cap progressively decreases in size as pigment streams to the interior of the cell.

Within 20 minutes, the entire gastrula or neurula is a heap of dissociated spherical cells which can be separated from one another with a glass needle. If the solution is now removed and replaced with 2–3 changes of Holtfreter's solution at pH 7.8, the entire process is reversed.

The cells begin to adhere to one another immediately after the removal of alkali, and within 0.5–1 hour have formed a mass of irregular form. At 3–8 hours, partly depending on species used, the aggregate has rounded up into a smooth solid ball of cells in intimate contact. Ectoderm cells, at first randomly distributed within the aggregate, have migrated to the exterior and formed sheet-like patches among the endoderm, at the end of 24–72 hours. If sufficient ectoderm cells are supplied they will almost completely cover the aggregate. Mesoderm cells are chiefly found between the ectoderm and endoderm, with a few scattered among the endoderm cells (Townes, unpubl.). The surface coat is reconstituted and the entire mass is usually covered with pigment. During this period intercellular spaces appear due to the secretion of an interstitial fluid. It appears to be identical with blastocoel fluid, having the same property of reducing the mutual adhesiveness of the cells (Holtfreter, 1944). Smaller aggregates lack the interior intercellular spaces altogether.

If an aggregate is allowed to develop further, differentiation proceeds and may include development of neural folds, eyes, ears, beating heart, etc. (Townes, unpubl.).

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Holtfreter (1939a, 1939b) has shown that small pieces of ventral ectoderm and endoderm, brought into contact, at first cling together but later tend to separate into discrete masses. The self-isolation begins after 2 days in *Rana pipiens* and 3–4 days in *Ambystoma punctatum*, and is complete after 4 and 6–9 days, respectively. However, if mesoderm is combined with ectoderm and endoderm, self-isolation does not occur; the mesoderm behaves as a binding agent.

These tissue affinities are apparently not species-specific at early stages of development, and may be seen in practically any xenoplastic combination. For example Townes (unpubl.) has found that the ectoderm of *Rana pipiens* gastrula with the endoderm of *Ambystoma punctatum* or *Triturus torosus* will exhibit self-isolation. If, however, mesoderm is included, an aggregate is formed which holds together.

With these observations and experiments as a basis it seemed desirable to test the effects of antisera on the reaggregation, extending thus the previously reported (Spiegel, 1954) experiments on dissociated sponge cells.

EFFECTS OF ANTISERA ON REAGGREGATION AND SEGREGATION

Materials and Methods

Experimental induction of breeding activity. Fertilized eggs of *Rana pipiens* were obtained through ovulation by hypophysis injection, and artificial insemination, as described by Rugh (1948). *Triton alpestris* eggs were very kindly furnished by Dr. J. Holtfreter.

Preparation of protein extract. Ten grams of the plant enzyme papain were extracted with 500 ml. of 10% Holtfreter's solution (3.5 grams NaCl, 0.05 gram KCl, 0.1 gram $CaCl_2$, 0.2 gram $NaHCO_3$, per liter distilled water) for six hours at 4° C. (Spiegel, 1951). The solution was filtered, 300 mg. cysteine.HCl added, and the pH adjusted to 6.6 with 0.1 *N* NaOH and 0.1 *N* HCl. Stage 12 (Shumway, 1940) gastrulas, 3410 in number, were placed in an eight inch finger bowl, the bottom of which was lined with ¼ inch of 2% agar, and covered with 500 ml. of the papain solution.

After three hours, the two outer layers of jelly had dissolved; the eggs were washed twice with 10% Holtfreter's solution, and 500 ml. of 2.5% sodium thioglycolate (in 10% Holtfreter's solution, pH 8.1) added. In ten minutes the vitelline membrane and third jelly layer were dissolved. The eggs were washed with three changes of 0.65% NaCl (0.01 *M* phosphate buffered at pH 7.3), without being allowed to come in contact with an air-water interface.

Protein was extracted in the medium used by Gregg and Ballentine (1946) (0.65% NaCl in 0.01 *M* phosphate buffer at pH 7.3), 65 ml. of solution being used for the 3410 eggs. The suspension was homogenized by hand for five minutes at 3–4° C. in a Ten Broeck glass homogenizer. A drop of the homogenate was examined under the microscope and no intact cells were noted.

The homogenate was kept at 4° C. for sixteen hours, with frequent shakings, then centrifuged at 600 G and the cloudy supernatant collected. Nutrient and blood agar plates streaked with the extract showed slight bacterial contamination. Therefore, 0.02 mg. Streptomycin ($CaCl_2$ complex) and 0.02 mg. Penicillin G potassium were added to each 50 ml. supernate to prevent bacterial growth and the extract was frozen in liquid air and stored at − 20° C. until use. The nitrogen

content of the extract, determined by micro-Kjeldahl, was 1.4 mg. N/ml. extract.

Twenty-four of 25 control eggs from the same clutch, and with jelly and vitelline membrane removed, developed normally to stage 22.

Preparation of antiserum. One male New Zealand Giant rabbit received eight subcutaneous injections, 0.5 ml. extract per injection, every fourth day (Cooper, 1948). A second uninjected male served as control. Seven days after the last injection, the animals were bled by cardiac puncture and the sera recovered. Both sera were heated to 56° C. for 0.5 hours to inactivate complement. Serum obtained from the animal injected with gastrula extract will henceforth be called anti-gastrula serum.

Precipitation tests were set up by layering 0.1 ml. of undiluted extract and of 11 two-fold dilutions (1:2 to 1:2048 in saline) over 0.1 ml. of antiserum, or over saline or normal serum as controls, in 10 × 75 mm. serological tubes. All tubes were incubated at 37° C. for one hour and then examined for presence of a precipitate ring at the interface. The antiserum produced rings of antigen-antibody complex at all antigen dilutions down through 1:1024. Controls were negative.

Tests of antiserum effects on reaggregation. For experiments dealing with ectoderm and endoderm, the membranes were removed with fine forceps from stage 10 or 11 *Rana pipiens* embryos, or the dorsal lip stage of *Triton alpestris,* and the desired tissues were isolated with glass needles in Holtfreter's solution. If mesoderm was to be used in addition to ectoderm and endoderm, the tissues were isolated either from stage 12 *Rana pipiens* embryos or from the dorsal lip stage of *Triton alpestris.*

The isolates were transferred by pipette to a syracuse dish containing 10 ml. sterile Holtfreter's solution, with 2% agar as substratum, and 1% KOH was added dropwise until disaggregation was complete. Occasionally, a clear viscous, somewhat elastic material or slime could be seen in the disaggregate; such preparations were rejected with one exception. The cells were washed 4–6 times with sterile Holtfreter's solution. Finally 10 ml. of normal serum or anti-gastrula serum, diluted with distilled water to give a final salt concentration of 0.38%, were added. By use of glass needles the cells were pushed into a heap with ectoderm, mesoderm, and endoderm cells randomly distributed.

Results

Twenty different combinations of tissues, isolated cells, antiserum, normal serum, and Holtfreter's solution were examined.

Effect of Rana pipiens anti-gastrula serum on disaggregated whole Rana pipiens embryos. Stage 9, 10 and 12 embryos were disaggregated and placed in anti-gastrula serum. After 1.5 hours the reaggregation, if any, was slight. At 7–14 hours the cells had adhered and sorted out to some degree, but remained spherical with conspicuous intercellular spaces. Endoderm cells in particular were loosely packed. Some slime was noted between the cells. In the stage 12 disaggregate, it appeared as though ectoderm cells would adhere to ectoderm, but not to endoderm, when pushed into contact. Endoderm cells, on the other hand, would adhere to endoderm, but not to ectoderm.

In contrast, the cells of disaggregates of stage 9 and 12 embryos, in *normal* serum, adhered to each other at the end of four hours, and the aggregate began

to round up. At eight hours, the aggregates were well rounded with interspersed patches of ectoderm and endoderm cells forming a smooth surface. At 15 hours the coat was being reconstituted. No masses of slime were noted.

Effect of Rana pipiens anti-gastrula serum on disaggregated ectoderm, endoderm, and mesoderm cells of Rana pipiens. After one hour in anti-gastrula serum, about 100 disaggregated mixed ectoderm, mesoderm, and endoderm cells, from a stage 12 embryo, had partially adhered. The ectoderm and endoderm cells appeared to have sorted out, but were loosely packed. No change was noted after this period.

Effect of antiserum on disaggregated ectoderm and endoderm cells of Rana pipiens. After 3.5 hours in antiserum, approximately 100 disaggregated ectoderm and endoderm cells, from a stage 11 embryo, adhered to each other, but the aggregate failed to round up. At four hours, the cells remained spherical and the aggregate surface was not smooth. Ectoderm and endoderm cells appeared randomly dispersed. No slime was noted. At 65 hours the ectoderm and endoderm cells still had not sorted out, and the surface was unchanged.

Ectoderm and endoderm cells of stage 11, after one hour in *normal* serum, had adhered and the surface was becoming smooth. By four hours, the aggregate had rounded up and ectoderm and endoderm cells had sorted out. The cells at the surface were flattened and a smooth exterior was noted at 17 hours. By 65 hours, the ectoderm and endoderm formed two separate aggregates connected by a short stalk of cells.

Effect of Rana pipiens anti-gastrula serum on disaggregated endoderm and ectoderm cells of Triton alpestris. Approximately 130 ectoderm and endoderm cells of the dorsal lip stage of *Triton alpestris* were disaggregated and placed in *Rana pipiens* anti-gastrula serum. After one hour, the cells had adhered to each other, with surface cells flattened and in intimate contact. At 17.5 hours the aggregate had become spherical with smooth surface. No slime was noted. At six

TABLE I

Summary of experiments showing effects of frog anti-gastrula serum on reaggregation of dissociated frog and salamander cells

	Mixtures of disaggregated ectoderm and endoderm cells				Mixtures of disaggregated ectoderm, mesoderm, and endoderm cells			
	Normal serum		Antiserum		Normal serum		Antiserum	
Condition	2R*	1T**	1R	1T	3R	1T	5R	1T
Time (hours)	17	17	28	18	8	16	14	16
Aggregates rounded:	+	+	−	+	+	+	−	+
Exposed cell surfaces are flattened:	+	+	−	+	+	+	−	+
Random intermixture of ectoderm and endoderm cells:	−		+		−		±	
Time (hours)	65	144	65	144	36	144	36	144
Ectoderm and endoderm in separate aggregates:	+	+	−	+	−	−	−	−

* Number of experiments with cells from *Rana pipiens* embryos (R).
** Number of experiments with cells from *Triton alpestris* embryos (T).

days the ectoderm and endoderm cells had sorted out to form two completely separate aggregates.

Ectoderm and endoderm cells in *normal* serum sorted out after six days to form two aggregates connected by a short stalk.

Effect of Rana pipiens anti-gastrula serum on disaggregated mesoderm, ectoderm, and endoderm cells of Triton alpestris. About 130 ectoderm, mesoderm, and endoderm cells of *Triton alpestris,* dorsal lip stage, in *Rana pipiens* anti-gastrula serum formed smooth surfaced aggregates after one hour and the aggregate became spherical by 16 hours. No slime was noted. Cells in *normal* serum behaved in identical fashion.

The results, summarized in Table I, indicate strongly that, in embryonic amphibian cells as in adult sponge cells, specific surface antigens are an essential part of the mechanism by which adjacent cell surfaces are bound together during reaggregation.

Discussion

The results with embryonic amphibian cells are difficult to explain except in terms of the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces similar to those between antigens and homologous antibodies (Tyler, 1940, 1942, 1946, 1947; Weiss, 1941, 1947, 1950).

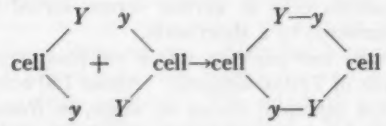
As in the case of cell adhesion in sponges described by Spiegel (1954) the results show that reaggregation of embryonic cells is inhibited, in contrast to the usual phenomenon of agglutination, by the presence of homologous antibodies. Spiegel (1954) has presented three conditions under which such inhibition may occur: (1) extreme antibody excess; (2) univalent antibodies; (3) structure of cell surface. Only the third condition will be discussed here (see Spiegel, 1954, for a discussion of conditions 1 and 2).

It has been suggested by Professor Albert Tyler that perhaps the cell surface of embryonic amphibian cells (and also of sponge cells) is a flexible, folded structure with the specific surface antigens so situated that the two or more valence groups of a *single* antibody molecule would tend to react with antigen on the *same* cell. Only a few, if any, valence groups would be free to react with receptor sites of other cells and thus would account for the absence of agglutination. It is, of course, also assumed that the reaction between specific surface antigens either blocks the adhesion sites or in some manner (*i.e.,* steric interference) interferes with them. The interpretation of the failure of the antibodies to agglutinate the sponge cells or the embryonic amphibian cells is a modification of the hypothesis presented by Coombs *et al.* (1951) to account for failure of homologous multivalent antibodies to agglutinate the red cells of most oxen. In their view a deep location of the antigens is assumed so that a multivalent antibody molecule after combining with one cell cannot reach receptors of a second cell.

That these surface antigens are specific has been demonstrated by the ability of cells of *Triton alpestris* embryos to reaggregate normally in *Rana pipiens* anti-gastrula serum.

The Tyler-Weiss hypothesis, as interpreted by Spiegel (1954), implies that adhesion depends on the presence, in the cell membranes, of two intra-molecular configurations with the reciprocal structural relationship of antigen and antibody. These exist either within a single molecular species or in distinct substances. If

the two reciprocal configurations are designated by Y and y, then the *unit* reaction in cell adhesion would be



As a logical consequence of this hypothesis, the segregation of ectoderm and endoderm cells from each other, in Holtfreter's solution or normal serum, into two separate aggregates, would indicate that different surface antigens are present on cells of each of the two tissues. Recent evidence obtained by Clayton (1953) with *Triton alpestris* embryos, seems to indicate that ectoderm and mesoderm, at least, contain different antigens. However, endoderm antigens were not investigated and the location of these antigens in the cell was not determined.

The fact that mixtures of ectoderm, endoderm, *and* mesoderm cells, in Holtfreter's solution or normal serum, round up to form a single aggregate consisting of three concentric layers of cells, with the mesoderm layer situated between ectoderm and endoderm (as in normal development), suggests an interesting possibility with regards to the surface antigens of mesoderm. Perhaps mesoderm, in addition to possessing specific surface antigens which are important for the adhesion of mesoderm cells to each other, also possesses sites which are complementary to ectoderm surface antigens *and* to endoderm surface antigens. Thus, in effect, mesoderm would act as a heterovalent antibody (a multivalent antibody molecule, with *different reactive sites,* which are capable of combining with at least two different antigenic groups) to ectoderm cells and to endoderm cells, and would be capable of binding the two cell types together. This would be analogous to the hypothesis proposed by Spiegel (1954) for the failure of segregation of mixtures of the cells of two species of sponge in antiserum produced in rabbits by simultaneous injection of cells of *both* species. Here it was proposed that *the rabbit* formed heterovalent antibody to the surface antigens of cells of both species and thus such antibody molecules were able to agglutinate both cell-species.

Each of the amphibian results is what would be expected from the sponge experiments performed by Spiegel (1954). Amphibian and sponge experiments, together, can be explained on the basis that the ability of cells to recognize each other depends upon the presence, in their surfaces, of specific antigens which also bring about the normal adhesion of cells, by reciprocal reactions similar to those between antigens and their homologous antibodies.

The author would like to express his gratitude to Prof. Donald R. Charles under whose guidance the work was carried out. He is also indebted to Prof. Albert Tyler for his aid in the editing of the manuscript.

Summary

An investigation of cell adhesion in embryonic amphibian cells is reported here. Antiserum was made in rabbits vs. a cell extract of stage 12 *Rana pipiens* embryos. The reaggregation of dissociated cells of Rana ectoderm and endoderm was in-

hibited by the antiserum. The antiserum had no effect on the reaggregation and segregation of dissociated cells of *Triton alpestris* embryos. The role of antigen-antibody-like reactions in cell adhesion is discussed.

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